Coenzyme Q redox signalling and longevity

Filippo Scialo[1], and Alberto Sanz[2].

[1] Dipartimento di Scienze Mediche Traslazionali, Università della Campania “Luigi Vanvitelli”, 80131, Napoli, Italia.

[2] Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, G12 8QQ, Glasgow, United Kingdom.

Correspondence: Prof Alberto Sanz, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, G12 8QQ, Glasgow, United Kingdom. Email: alberto.sanzmontero@glasgow.ac.uk

Abstract

Mitochondria are the powerhouses of the cell. They produce a significant amount of the energy we need to grow, survive and reproduce. The same system that generates energy in the form of ATP also produces Reactive Oxygen Species (ROS). Mitochondrial Reactive Oxygen Species (mtROS) were considered for many years toxic by-products of metabolism, responsible for ageing and many degenerative diseases. Today, we know that mtROS are essential redox messengers required to determine cell fate and maintain cellular homeostasis. Most mtROS are produced by respiratory complex I (CI) and complex III (CIII). How and when CI and CIII produce ROS is determined by the redox state of the Coenzyme Q (CoQ) pool and the proton motive force (pmf) generated during respiration. During ageing, there is an accumulation of defective mitochondria that generate high levels of mtROS. This causes oxidative stress and disrupts redox signalling. Here, we review how mtROS are generated in young and old mitochondria and how CI and CIII derived ROS control physiological and pathological processes. Finally, we discuss why damaged mitochondria amass during ageing as well as methods to preserve mitochondrial redox signalling with age.

Highlights

- mtROS are active redox messengers that participate in essential cellular processes ranging from cell differentiation to tissue homeostasis.
- Most mtROS are produced by CI and CIII. CI and CIII derived ROS regulate specific physiological and pathological processes.
- The redox state of the Coenzyme Q pool determines electron leak and mtROS production by CI and CIII.
- During ageing, an accumulation of defective mitochondria generates vast amounts of mtROS. These ROS cause oxidative damage and interfere with redox signalling.

List of Abbreviations

ATPIF1	ATPase Inhibitory Factor 1
AOX	Alternative oxidase
CI	Complex I, NADH:ubiquinone oxidoreductase
CII	Complex II, succinate:ubiquinone reductase
CIII	Complex III, ubiquinol:cytochrome c oxidoreductase
CIV	Complex IV, cytochrome c oxidase
CO	Carbon monoxide
ETC	Electron transport chain
CoQ	Coenzyme Q, ubiquinone
$CoQ^{\cdot}$	Semi-ubiquinone
CoQH2	Ubiquinol
CV	Complex V, ATP synthase
ETC	Electron transport chain
H_2O_2	Hydrogen peroxide
hiROS	High Reactive Oxygen Species
HO-1	Heme-oxigenase-1
HO-2	Heme-oxigenase-2
$HO^{\cdot}$	Hydroxyl radical
IF	Flavin site of CI
IQ	Quinone-binding site of CI
IIIQo	External quinone site of CIII
loROS	Low Reactive Oxygen Species
LPS	Lipopolysaccharides
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtROS	Mitochondrial ROS
NDI1	NADH dehydrogenase internal 1
NO	Nitric oxide
NOS	Nitric oxide synthase
NOXs	NADPH oxidases

$O2^{\cdot-}$	Superoxide
OXPHOS	Oxidative phosphorylation
pmf	Proton motive force
RC	Respiratory chain
RET	Reverse electron transport
RISP	Rieske iron-sulphur protein
ROS	Reactive Oxygen Species
S1QELs	Suppressors of site IQ Electron Leak
S3QELS	Suppressors of site IIIQo Electron Leak
XO	Xanthine oxidase

1. Introduction

1.1. Mitochondria and ageing: the unavoidable decay of the cellular powerhouse.

Mitochondria are popularly known as the “powerhouses” of the cell, referencing their role in the production of cellular energy. Along with producing the majority of cellular energy they are also required for many vital biosynthetic processes instrumental for survival, such as the synthesis of iron-sulphur clusters, CoQ and pyrimidines. Mitochondria are also signalling hubs that generate essential cellular components, regulate metabolism, and control cell fate. In the process of aging mitochondria become less efficient, producing less ATP and more Reactive Oxygen Species (ROS). The accumulation of these defective mitochondria is a universal hallmark of ageing observed across cell types and species [1]. Given the essential role mitochondria play in cellular metabolism it is unsurprising that age-associated loss of mitochondrial function has been linked with onset of many age-related diseases such as cancer, diabetes and Parkinson's disease [2]. We do not understand how or why defective mitochondria accumulate with age or how we could prevent, delay, or reverse this. Identifying approaches to maintain mitochondrial homeostasis will help to increase health span and delay the onset of devastating age-associated diseases.

The rise in mitochondrial ROS (mtROS) with age correlates with increased levels of oxidative damage in the form of oxidised proteins, lipids and DNA [3]. For many years, the accumulation of oxidative damage was considered the leading underlying cause of ageing [4]. Now it is accepted that neither ROS nor oxidative damage drive ageing but consequence of it and as such contribute to the onset of age-associated disease [5-7]. This explains why both supplementation and genetic overexpression of antioxidants fails to extend lifespan in animal models [3, 8] or positively impact human health in controlled clinical trials [9]. High levels of mtROS cause cellular alterations and compromise health span. The failure of antioxidant therapies to prevent this indicates that the effects of excess mtROS are complex and cannot be explained solely by oxidative stress and the accumulation of oxidative damage. One often missing variable in this equation is the role ROS play as signalling molecules. ROS participate in three processes that determine cell fate, namely cell division, differentiation/growth, and death. Interruption of mtROS signalling can therefore have potentially severe consequences on survival. For example, moderate levels of mtROS, produced in a controlled manner, are required for differentiation of haematopoietic progenitors in *Drosophila* [10], exercise induced muscle adaptation in humans [11] and activation of the inflammasome in response to infection [12]. A massive increase in mtROS concentration, such as that observed during ageing [13], not only increases oxidative damage but also severely alters redox signalling and

consequently cellular fate. There is plenty of evidence in the literature of high levels of mtROS triggering oxidative stress and subsequently oxidative damage. A much less investigated topic is how increased production of mtROS may interrupt redox signalling, although interest in understanding this is growing and is the topic of this review. Here, we aim to review the latest research on mtROS, taking into consideration where and when ROS are produced, how they participate in redox signalling, contribute to cellular homeostasis and the consequences of mtROS signalling dysregulation. We will focus on ROS produced by mitochondrial CI and CIII. CI and CIII are the two ROS generators which have been studied most *in vivo* [14]. The production of ROS, at CI and CIII, is directly related to the reduction status of the mitochondrial CoQ pool. We will review how changes in the redox state of CoQ determine the electron leak from CI and CIII. Finally, we will discuss how age-associated loss of mitochondrial activity affects redox signalling and discuss strategies to preserve mtROS signalling in aged individuals.

1.2. The Electron Transport Chain: imperfect machine or sophisticated communication system?

In our revision of the literature, we will focus on mtROS and their role in ageing. There are ROS generators located outside the mitochondrion and they may contribute to ageing. We will refer to them when appropriate but they will not be discussed in detail as there are already many excellent reviews on this topic [15, 16]. Within mitochondria, the respiratory chain (RC) is the main site of ROS production, although ROS are also produced at sites outwith the electron transport chain (ETC) [17]. The RC synthesises energy in the form of ATP, with the majority of mitochondrial functions coupled to the uninterrupted transfer of electrons through ETC. For example, pyrimidine biosynthesis [18] or histone acetylation [19] are seriously affected if electron transport is interrupted. The RC is made of five respiratory complexes. Respiratory complexes I to IV are part of the ETC and work closely coupled to the function of respiratory complex V (CV, *aka* ATP synthase), which completes oxidative phosphorylation (OXPHOS). ETC complexes I (*aka* NADH:ubiquinone oxidoreductase) and II (*aka* succinate: ubiquinone reductase, CII) introduce electrons into the ETC, while complexes I, III (*aka* ubiquinol: cytochrome c oxidoreductase) and IV (*aka* cytochrome c oxidase, CIV) use the energy released by the transfer of electrons to pump protons into the intermembrane space. The pumping of protons to the intermembrane space generates a proton motive force (pmf), which is used by CV to phosphorylate ADP to ATP, with energy produced during the transport of protons back to the matrix. Pmf has two components one chemical, i.e. pH, and one electrical, i.e. the charge differential between the intermembrane space and matrix, known as the membrane potential. As we will see, pmf is one of the critical factors which determine

electron leak and the subsequent ROS production by ETC. The difference in pH (∆pH) between the intermembrane space and the mitochondrial matrix is extraordinarily complicated to measure *in vivo* and so membrane potential is used as a proxy measurement for pmf [20]. Other than CI and CII, dehydrogenases including glycerol-3-phosphate dehydrogenase, electron-transferring flavoprotein ubiquinone oxidoreductase and dihydroorotate dehydrogenase can introduce electrons into ETC and contribute to the generation of pmf.

All electrons which enter the ETC are transferred to a molecule of ubiquinone (CoQ) which becomes ubiquinol ($CoQH_2$) when reduced by two electrons. $CoQH_2$ transfers electrons to CIII, and CIII gives them to cytochrome c. As we will see, the redox state of CoQ (i.e. the ratio of $CoQH_2$ to CoQ) is the other primary determinant, together with the ∆pH, of electron leak within the ETC. Cytochrome c transfers electrons to respiratory CIV. In aerobic organisms, the final acceptor of electrons, which do not have enough energy to facilitate proton pumping, is oxygen. Oxygen is reduced by CIV with four electrons and two protons. Most electrons that enter the ETC end up participating in the reduction of oxygen to water by CIV. However, a small fraction of them, between 0.1-2% depending on the experimental conditions, leak out of the ETC before reaching CIV and reduce oxygen directly producing mainly superoxide that is immediately dismutated to hydrogen peroxide by SOD1 or SOD2 [21]. Historically, mtROS were considered as unavoidable by-products of metabolism [22] and the ETC as imperfect and unable to operate without producing “pollutant” free radicals. This has now been shown to be incomplete, with mtROS now seen as essential cellular messengers and the ETC a sophisticated communication system that produces redox signals in response to metabolic alterations [23]. Current research is not focused on preventing all ROS production, but in understanding when and how it should be stimulated to favour redox signalling as well as identifying ways to specifically inhibit unwanted electron leak.

In ageing, there is a decrease in mitochondrial respiration that is conserved across species and has been described in various cell types and tissues [24-28]. The age-associated alteration in mitochondrial respiration has two consequences. It increases levels of mtROS and alters other processes associated with the ETC such as synthesis of pyrimidines or iron-sulphur clusters [29]. A number of studies report that CI is more affected than other respiratory complexes, with its activity reduced principally and more quickly than the activity of other respiratory components [13, 27, 30]. This could be explained by CI having, a greater number of subunits than the other ETC complexes, a more complicated assembly process and that the majority of its subunits are exposed to ROS generated within the matrix [31, 32]. In line with this, turnover of CI subunits is faster than that of proteins part of other respiratory

components both in *Drosophila melanogaster* [33] and in mouse cell culture [32]. Autophagy is decreased during ageing [34], the faster turnover required by CI subunits could contribute to the more profound decline in CI activity observed in ageing and age-related diseases [13, 30]. As we will analyse, the age-associated decline of CI can affect ROS production and the essential signalling pathways that they control.

2. The conflicting effects of mtROS on ageing

2.1 Low versus High Reactive Oxygen Species: signalling versus damage.

One reason why the field of redox biology has not advanced as quickly as anticipated is that for many years all ROS were considered as a singular "bad" entity. Now, it is understood that ROS are not all the same. There are numerous types of ROS, with differing reactivities and half-lives [15]. Superoxide ($O_2^{\cdot-}$), for example, is a free radical and therefore more reactive than non-free radical ROS such as hydrogen peroxide (H_2O_2). Superoxide and hydrogen peroxide are the most abundant and most studied ROS *in vivo* [8]. They are produced when oxygen is reduced with either one or two electrons. They will be the focus of this review and the term ROS will refer to either superoxide and/or hydrogen peroxide unless otherwise noted. Other than superoxide and hydrogen peroxide, the hydroxyl radical ($HO^{\cdot}$), the result of reducing oxygen with three electrons is often cited as critical for ageing. Despite this it is rarely directly measured in ageing studies due to its high-reactivity and short half-life and is usually estimated using various proxy measurements of oxidative damage such as levels of lipid oxidation or protein carbonylation. ROS can be divided into two categories according to their reactivity: (i) low Reactivity Oxygen Species (loROS) and (ii) high Reactivity Oxygen Species (hiROS). Superoxide and hydrogen peroxide are examples of loROS, whereas the hydroxyl radical is an example of hiROS.

loROS have a limited capacity to directly attack lipids, proteins and nucleic acids [29]. Cells possess a complex system of enzymatic antioxidants that specifically neutralise superoxide and hydrogen peroxide. Superoxide is immediately dismutated to hydrogen peroxide by SOD1 or SOD2 [29], while hydrogen peroxide is detoxified by different set of enzymes depending on where and how much is generated. These include catalases, glutathione peroxidases and peroxiredoxins. hiROS, such as the hydroxyl radical, directly attack biological molecules. It is unclear if the hydroxyl radical is ever produced as a by-product of oxidative phosphorylation, or instead, as a result of the reaction of either superoxide or hydrogen peroxide with free metals (e.g. Fe^{2+}) through the Fenton and Haber-Weiss reactions [29]. Other examples of hiROS are singlet oxygen, peroxynitrite and hypochlorous acid. Outwith certain exceptions, such as hypochlorous acid which is produced by neutrophils to fight a bacterial infection, how

often hiROS are produced in non-pathological conditions is unclear. The lack of specific antioxidant systems targeting hydroxyl radicals suggests that hiROS production is rare or unpredictable and therefore there was likely no evolutionary advantage to developing a highly specific, but energetically expensive, detoxification system. It is important to emphasize that this contrasts with loROS where specific antioxidant systems allow control of loROS levels. Non-enzymatic antioxidants such as vitamin C, E and uric acid can protect against the effects of hiROS. From a signalling standpoint, antioxidants could acts as modulators and even transducers of ROS signals [35]. For instance, catalases can eliminate a hydrogen peroxide signal, while peroxiredoxins can oxidise specific cysteine residues within proteins with electrons from hydrogen peroxide, transmitting information in the process [36]. In fact, the "redox-relay" model proposes that peroxiredoxins act as transducers of information recognising and oxidizing specific target proteins that control redox-modulated signalling pathways [37]. Alternatively, the "floodgate" model states that peroxiredoxins "only" allow transmission of information when hyperoxidized [38]. Most likely, both models are partially correct and peroxiredoxins participate in redox signalling acting both as "relays" and "gates" depending on the physiological context.

In vivo, hiROS are most likely produced by reactions of loROS with free transition metals. An excellent example of this is mitochondrial superoxide attacking iron-sulphur clusters of CI, CII and aconitase, producing hydroxyl radicals through the Fenton/Haber-Weiss reactions [29]. In *pink1* mutant flies, high levels of mtROS result in the release of free iron from aconitase increasing oxidative damage and causing substantial behavioural and morphological alterations [39]. These deleterious phenotypes are supressed when iron-sulphur clusters have been eliminated from aconitase using site-specific mutagenesis. Similarly, high levels of superoxide can react with nitric oxide to produce peroxynitrite that inactivates proteins through amino acid nitration, which is an oxidative stress marker found in the brain of Alzheimer's patients [40].

As we will discuss in detail, one consequence of the accumulation of damaged mitochondria during ageing is an increased level of hiROS due to the very high levels of superoxide and hydrogen peroxide produced by "old/dysfunctional" mitochondria. This triggers oxidative stress and supresses redox signalling by diminishing the capacity of the cell to differentiate specific mitochondrial ROS signals. For any signal to be effective, it must be produced in response to specific stimuli and with the appropriate strength. With redox signalling this is achieved by the site specificity of production and the amount of ROS. Thus, it is essential that the signal can be switch on and off to maximise the transmission of information. Although both superoxide and hydrogen peroxide have been proposed as redox messengers, the latter is theoretically the stronger candidate as it is not a free radical, is more stable and uncharged.

Therefore it can diffuse further from the site of production, including through lipid membranes [41]. Accordingly, the Sanz's laboratory has recently shown that mitochondrial hydrogen peroxide is necessary for stress adaptation in *Drosophila melanogaster* [42]. When mitochondrial hydrogen peroxide levels are drastically reduced through expression of a mitochondrially-targeted catalase, these flies are short lived under both thermal and oxygen stress, hypoxia and hyperoxia. Reducing levels of mitochondrial superoxide without decreasing mitochondrial hydrogen peroxide did not result in reduced survival under stress. Similarly, a mitochondrially-targeted catalase expressed in mouse glial cells causes significant dysregulation of neuronal metabolism and cognitive alterations [43], demonstrating that hydrogen peroxide signalling is instrumental for optimized brain function.

Finally, evidence from many independent laboratories in various model organisms illustrates that enhancing mtROS levels through chemical or genetic disruption of the ETC contributes to extension of cellular homeostasis and lifespan in worms, flies and possibly also in fish and mice [8, 44]. This phenomenon has been named mitohormesis, based on the hypothesis that the same mechanisms that protect against oxidative stress are activated to protect the organism and extend lifespan [45]. It is unclear what the exact mechanisms that extend lifespan when mtROS are experimentally manipulated are. Initially, antioxidants were considered as the canonical candidates, however overexpression of antioxidants *per se* does not prolong lifespan [3] and overexpression of antioxidants in animals where mtROS have been increased suppresses the lifespan extension effects of mtROS [46, 47]. Despite the positive effects of increasing mtROS on longevity, we should keep in mind that when mtROS or any other ROS are in excess, in the wrong place and for too long, they are dangerous and can have negative consequences on cells and tissues [48] as well as on the lifespan of the organism [49].

2.2. *In vitro* versus *in vivo* ROS: a problem of resolution.

Another significant barrier to progress in understanding the redox biology of ageing is the complexity of estimating mtROS concentrations *in vivo*. Until recently identifying the site of mtROS production was extremely challenging. For years mtROS levels were measured using isolated mitochondria supplemented with saturating concentrations of substrates, to feed the ETC, as well as inhibitors specific to each respiratory complex to interrupt electron flow [8]. This approach allows high resolution and therefore the identification of the source and mechanism underlying mtROS production. Despite this, it has many drawbacks. Measurements are taken in supraphysiological oxygen levels, in the absence of contact with the cytosol and other organelles. They rely on unphysiological saturating concentrations of substrates to direct electron entry specifically through CI or CII, either in the absence of or saturation of ADP, detect only hydrogen peroxide leaked by mitochondria and artificially alter

the redox state of the ETC due to the use of inhibitors. Together these call into question the physiological relevance of measurements in isolated mitochondria but we cannot completely dismiss work carried out with isolated organelles. We certainly observe differences in mtROS production between populations of mitochondria isolated from different animals or individuals, indicating functional or morphological differences between them. Mitochondria isolated from livers of protein-restricted rats produce less hydrogen peroxide [50]. Western-blot analysis shows that mitochondria from protein-restricted rats have lower levels of CI, explaining the lower levels of mtROS [51]. Similarly, pigeon heart mitochondria produce fewer mtROS and have lower levels of CI when compared to rat mitochondria [52]. In mouse fibroblasts, reduction of CI, by promoting degradation of the complex, results in decreased levels of mtROS [32]. In conclusion, measurements in isolated mitochondria are useful when used in combination with other approaches like mass spectrophotometry to uncover and understand the functional and morphological differences of mitochondria or when comparing different individuals, species or experimental conditions.

Mitochondria isolated from long-lived individuals or species produce fewer mtROS. In most cases these differences are only apparent under certain conditions, when CI-linked substrates (e.g. pyruvate, glutamate, proline) or succinate (without rotenone), that induces reverse electron transport (RET), are used [53]. Many of these studies do not measure CI and CIII derived ROS in similar conditions. Usually, the concentration of succinate (+rotenone) to study CIII ROS is between four and five times higher than the concentration used with CI-linked substrates. Under these conditions, CIII becomes highly reduced (i.e. more than CI) possibly masking differences in electron leak [54]. It is possible that using lower concentrations of succinate, CIII would also produce fewer ROS in long-lived animals/individuals. CI and CIII are not the only producers of ROS within mitochondria, in mitochondria isolated from rat skeletal muscle up to 11 different ROS generators have been identified [55]. These might only produce ROS when saturating concentrations of substrates are used and/or when electron transfer is inhibited by specific drugs. Accordingly, when saturating substrates and inhibitors are replaced by an *ad hoc* media that mimics cytosolic conditions found in muscle during either rest or exercise, they find that main generators of ROS are CI, CII and CIII [56]. In the previous study, the authors estimate mtROS levels using two methods. The classical peroxidase/Amplex Red protocol, where Amplex Red is oxidised by hydrogen peroxide, and endogenous reporters which allow indirect quantification of ROS levels from either the flavin site of CI (IF) or the external quinone site of CIII (IIIQo) [57]. The endogenous reporters estimate the generation of superoxide from IF and IIIQo by following changes in the redox state of NAD(P)H and cytochrome b655 respectively. They evaluate ROS produced specifically by CI and CIII without using inhibitors and avoiding some of the disadvantages of

the Amplex Red/peroxidase system [58]. Use of endogenous reporters or Amplex Red/peroxidase produce comparable results. Under conditions that simulate those observed in resting muscle, the quinone-binding site of CI (IQ) and IF produced most of the ROS. However, when conditions simulate muscle during exercise, ROS production is decreased at all sites and IF is the main site of ROS production [57]. For the most part *ex* and *in vivo* studies support that CI and CIII are the main sources of ROS, with CII playing an essential role in certain cancers resulting from mutations in CII subunits [59].

Mitochondria are believed to be the main generators of ROS in cells. The process of OXPHOS is the primary consumer of cellular oxygen and continually working to produce ATP. However, as we mentioned, most work investigating mtROS production has been carried out in isolated mitochondria, making an equal comparison with other sources of ROS unfeasible. Measuring mtROS in cell culture is an alternative that allows assessment of how much ROS comes from mitochondria in comparison to the rest of the cell. It avoids all the caveats outlined above with the exception of supraphysiological oxygen concentrations. This could be avoided by culturing cells under physiological oxygen conditions using a hypoxic chamber, but cells are routinely cultured in 20% environmental oxygen and not under normoxic conditions, which results in specific adaptations e.g. cells adapt by becoming extraordinarily glycolytic. These alterations mean cells behave very differently from how they would *in vivo* [60]. Studies using cells or tissues usually only measure total ROS or ROS produced by mitochondria and only a few published studies investigate the different generators of ROS in the same experimental conditions. The laboratory of Martin Brand has investigated where and how much ROS are generated in cells cultured *in vitro*. They have shown that in myoblasts (C2C12), mitochondria produce no more than 50% of total hydrogen peroxide [61]. They measured the total amount of peroxide leaking from the cell since the Amplex Red/peroxidase system does not enter the cell. The proportion of total ROS produced by mitochondria does not change when myoblasts differentiate to myotubes. In a recent publication [62], Brand's laboratory extend this analysis with several more cell types including primary and immortalised cell lines from seven different tissues (bone, cervix, heart, liver, lung, neuronal and skin) derived from both rodents and humans. They observed variability in the amount of ROS produced by mitochondria in different cell lines but did not report a single case where mitochondria produced more than 50% of total ROS. In the majority of cell lines studied, significant ROS were produced by NADPH oxidases (NOXs) [62]. It is possible that mitochondrial peroxide is largely neutralised before leaking the cells, and therefore these measurements underestimate the contribution of the mitochondria. These studies also do not consider superoxide that is not detoxified by superoxide dismutases, that may contribute to oxidative damage. In any case, we should not discount the amount of superoxide and mainly hydrogen peroxide which is produced by other organelles, such as the

endoplasmic reticulum and peroxisomes, and individual enzymes, such as NOXs, monoaminoxidases, cyclooxygenases and nitric oxide synthases (NOS). NOXs are the single most important generators in most of cell lines studied by the Brand lab [62], and peroxisomes produced more hydrogen peroxide than mitochondria in hepatocytes isolated from rats [63].

As we have outlined, understanding where exactly ROS are produced within mitochondria is extremely important to be able to manipulate mtROS levels to extend health span. In our field, a common approach to dissecting the source of ROS can be summarised in four steps (Figure 1). Firstly, identify a phenotype to study, e.g. differences in lifespan between two different groups. Secondly, measure mtROS levels and determine the nature of the relationship between mtROS levels and the phenotype under study, in our example lifespan. Thirdly, inhibit mtROS production by the suspected generator using chemical and/or genetic approaches. Finally, to confirm the role of the mtROS generator on lifespan, use over-expression of or supplementation with antioxidants to supress the effects on longevity. If possible, induce ROS through an alternative mechanism and test whether the phenotype is recapitulated. These steps are essential to be able to confirm how site specific mtROS signals regulate physiological phenotypes. However, some steps can be difficult and are omitted. For example, inhibitors required to block site specific ROS production are restricted to ROS measurements as they can be toxic in animals preventing their use in lifespan studies. This four-step approach is used routinely nowadays, but it is not without critics. The main criticism cites that inhibition of one site in the ETC will increase (or decrease) ROS leak upstream (or downstream) [61]. Therefore, observed decreases in ROS or phenotypic effects may be a result of these accessory changes in ROS and not of the intended modification.

An alternative to the use of a selective ETC inhibitor, is the use of molecules that selectively trap electrons which leak from specific ROS producers without interrupting mitochondrial respiration. Suppressors for the IQ and the IIIQo sites are unsurprisingly called Suppressors of site IQ Electron Leak (S1QELs) and Suppressors of site IIIQo Electron Leak (S3QELS) [64]. S1QELs trap electrons leaked from IQ before they can react with oxygen. S3QELs do the same but with electrons from IIIQo. The use of S1QELs and S3QELs in combination with specific inhibitors of other ROS-generators allows estimation of the contribution of CI and CIII to cellular ROS levels [61]. Like similar molecules, the use of S1/3QELs is not without caveats, particularly *in vivo*. In animals, it can be difficult to guarantee that S1/3QELs reach the right site and exclusively that site, and if they do that they work as expected.

3. CoQ, a master regulator of redox signalling

3.1 The redox state of CoQ regulates CI/CIII ROS production

Electron leak at respiratory CI and CIII is strongly influenced by the redox state of the CoQ pool. CoQ is a two-electron carrier universally present in lipid membranes of plants, animals, fungi, and bacteria. In the mitochondrion, it receives all electrons entering the ETC and transfers them to CIII. CoQ can hold three redox states: oxidized (ubiquinone, CoQ), semi-oxidized with one electron (semi-ubiquinone, $CoQ^{\cdot}$) or reduced with two electrons (ubiquinol $CoQH_2$) (Figure 2). Both $CoQH_2$ and CoQ are stable and non-radical forms, but $CoQ^{\cdot}$ is a free radical and can donate its unpaired electron to oxygen, forming superoxide. This happens both at the IQ and IIIQo site [65]. The redox state of CoQ is determined by the entry and exit of electrons into ETC. A high $CoQH_2$: CoQ ratio increases the half-life of $CoQ^{\cdot}$ and favours the formation of superoxide [21]. Accordingly, the $CoQH_2$:CoQ ratio, together with pmf, are the determining factors of electron leak from the ETC and therefore of mtROS production [66]. The $CoQH_2$:CoQ ratio has been proposed to act as a sensor of mitochondrial function and transmitting this information through a ROS-RET signal produced by CI [32, 67].

CI produces ROS in both the forward and reverse direction [14]. ROS production in the reverse direction was first described by Britton Chance in the 1960s [68]. Chance reported the generation of NADH from NAD+ in isolated mitochondria upon succinate supplementation. NADH is generated thanks to RET from $CoQH_2$ to CI. Interestingly, during RET, a significant amount of ROS is generated by CI. It is widely accepted that the IF site produces ROS when electrons are transported in the forward direction and inhibitors of the IQ site are used to block the exit of electrons [21]. However, it is highly controversial at which site ROS are produced during RET [69]. Some studies support IF as the only site of ROS production during both forward and reverse electron transport [70, 71], while others propose that during forward electron transport , IF is the site of electron leak, whereas during RET IQ is the site of electron leak [55]. S1QELs, that specifically trap electrons preventing leaking from the IQ site, have recently been shown to inhibit ROS-RET in several physiological settings such as ischemia-reperfusion, supporting the idea that IQ is the site of electron leak during RET [64]. Irrespective of where ROS are produced in CI, inhibition of CII suppresses ROS-RET [48], suggesting that CII is necessary for RET. Other dehydrogenases that feed the ETC by reducing CoQ to $CoQH_2$ are also potentially involved in generating ROS-RET. Accordingly, it has been shown that feeding electrons to ETC via glycerol-3-phosphate dehydrogenase [72], the electron-transferring flavoprotein [32] or dimethylglycine dehydrogenase [73] triggers ROS-RET *in vitro*.

ROS-RET depends on two factors: (i) the redox state of CoQ, and (ii) pmf [13, 66]. When either of these is abolished or sufficiently reduced RET cannot occur and ROS levels return to normal. *In vitro* studies using isolated mitochondria show that around 60% of the CoQ pool needs to be reduced for ROS-RET to occur. Expression of the alternative oxidase (AOX) from *Ciona intestinalis* decreases the redox state of CoQ by around 20%, without affecting

membrane potential, and reduces ROS levels more than 100% [66]. *In vivo* expression of the alternative NADH dehydrogenase 1 (NDI1) from *Saccharomyces cerevisiae* increases the redox state of CoQ in the fly brain by approximately 15%, this mild increase is sufficient to trigger ROS-RET [13]. Co-expression of NDI1 and AOX prevented both the increase in levels of $CoQH_2$ and ROS.

ROS-RET is also regulated by pmf. For RET to occur a pmf high enough to provide energy for electron transfer from $CoQH_2$ to CI is required. It is unclear how the conditions required for ROS-RET arise *in vivo*. Diverse scenarios are supported by different *in vivo* models of ROS-RET. During ischemia-reperfusion, two events precipitate ROS-RET [48]. First, there is an accumulation of succinate during the ischemic phase due to the interruption of electron flow caused by oxygen deprivation. Second, this accumulated succinate is rapidly oxidised by CII during reperfusion which causes both over-reduction of CoQ, as well as an elevated pmf, that produces RET. Accordingly, inhibition of CII prevents an increase in ROS and protects against damage generated during ischemia-reperfusion [48]. Similarly, specialised cells of the carotid body, which detect changes in oxygen levels in the blood, have higher levels of succinate than other neurons. This facilitates triggering ROS-RET under conditions of hypoxia [74]. Macrophage reprogramming in response to bacterial stimuli is mediated by ROS-RET. Under these conditions, ROS-RET is achieved by inhibition of CV, which increases pmf, and increasing succinate oxidation, which increases $CoQH_2$ reduction [75]. In the brains of fruit flies, expression of NDI1 minimally increases electron entry into the ETC [76]. However, this modest increase is sufficient to increase the redox state of CoQ and trigger ROS-RET [13]. In the absence of NDI1, ROS-RET is initiated in brains of wild-type flies, when exposed to thermal stress i.e. incubation from 25 to 32 °C. We can hypothesize that the mechanism that triggers RET under thermal stress relies on increased substrate oxidation by the ETC that occurs under these conditions [77].

CIII is a major source of ROS within the ETC. Within CIII, ROS are generated at the IIIQo site [78]. The reason why IIIQo is a hotspot for electron leak is the way electron transfer occurs through the CIII Q-cycle. Within the complex, the electrons carried by $CoQH_2$ are split in two, one goes to the Rieske iron-sulphur protein (RISP) and the other to cytochrome b_L. RISP donates electrons to cytochrome c1, while cytochrome b_L contributes to the reduction of CoQ to $CoQH_2$ via cytochrome b_H. As in CI, there is univalent transfer of electrons, with the Q-cycle requiring $CoQ^{\cdot}$ formation to be completed. $CoQ^{\cdot}$ is usually immediately reduced with an electron provided by the oxidation of $CoQH_2$. However, when there is a block in the ETC or increased reduction state of some downstream components, slowing electron flow, $CoQ^{\cdot}$ tends to reduce oxygen to superoxide instead. For example, blocking IIIQi with antimycin A prevents further reduction of $CoQ^{\cdot}$ and triggers the generation of superoxide [79]. *In vivo*, situations that

increase the reduction of the CoQ pool or downstream molecules involved in electron transfer, boost the production of superoxide within CIII. An excellent example of this is how cellular hypoxia results in ROS production from the IIIQo [80]. A recent paper by Pablo Hernansanz-Agustin and co-workers dissects in detail the molecular mechanisms that trigger ROS production from CIII during hypoxia [81]. Firstly, hypoxia-induced deactivation of CI causes acidification of the mitochondrial matrix, which subsequently increases mitochondrial free Ca^{2+}. This rise in Ca^{2+} activates the exchange of mitochondrial Ca^{2+} for cytosolic Na^{2+}. Mitochondrial Na^{2+} reacts with phospholipids of the inner membrane, decreasing membrane fluidity and slowing movement of CoQ within the inner membrane. This presumably increases the half-life of $CoQ^{\cdot}$ and favours its reaction with oxygen to produce superoxide. Similarly, circumstances which favour the formation of $CoQ^{\cdot}$ at IIIQo by increasing the reduction of the CoQ pool or reducing electron flow downstream of CIII will promote ROS production.

3.2 Experimental manipulation of the redox state of the CoQ pool

As we have seen, the redox state of the CoQ pool determines the amount of ROS produced by CI and CIII. Therefore, experimentally manipulating the ratio $CoQH_2$:CoQ, particularly *in vivo*, should be a priority within the redox biology field. Increasing or decreasing the oxidation of CoQ in isolated mitochondria is quite simple. It can be achieved by using saturating concentrations of succinate to increase the redox state of the CoQ pool, triggering ROS-RET [66] or by using inhibitors to interrupt the electron flow, increasing or decreasing the amount of reduced/oxidised CoQ, e.g. blocking IIIQi with antimycin A triggers ROS production by stabilising $CoQ^{\cdot}$ [82]. *In vivo*, the ETC receives electrons from multiple substrates at different entry points; thus, both the substrates and entry points are different in distinct physiological states (e.g. rest *versus* exercise) and cells types (e.g. neurons versus glia) [43, 56]. Using ETC inhibitors is also complicated by the difficulties in ensuring delivery and function when used in animals, while inhibitors may be toxic at concentrations required to stop electron flow. Finally, interrupting electron flow not only alters the redox state of CoQ but also affects the oxidation state of many up and downstream ROS generators.

As we have mentioned, using S1QELs and S3QELs it is possible to specifically trap electrons at the IQ and IIIQo sites, respectively. Like traditional inhibitors, it can be challenging to specifically target the S1QELs/S3QELs to the required cells. The use of the so-called "alternative respiratory enzymes" allow evasion of these caveats. Alternative respiratory enzymes are so called as they are not expressed within the human ETC but are standard components of the ETC of plants, bacteria, fungi and some animal species [83]. Two of these enzymes, the already mentioned AOX and NDI1 have been extensively used to by-pass blocks in the ETC, alter the redox state of CoQ and the ROS generation [84] (Figure 2).

Like CIV, AOX accepts electrons from the CoQ pool and reduces oxygen to water with four electrons and two protons. AOX contributes to maintaining the oxidised state of the CoQ pool, reducing both electron leak and ROS levels [85]. When it is active, AOX by-passes both CIII and CIV [86] and has been shown to reduce ROS from both CI, preventing ROS-RET [66] and CIII [79]. AOX activity depends on the reduction level of the CoQ pool. When the CoQ pool is "over-reduced" AOX is active and will compete with CIII for electrons [87]. There are several classes of AOX enzymes, with differing affinities for both oxygen and $CoQH_2$. Each class of AOX is expressed or active in different physiological situations [88]. In mammalian cells, AOX from the fungi *Aspergillus nidulans* [89] and the ascidian *Ciona intestinalis [86]* have been expressed. However, the majority of studies in animals has been performed with *Ciona* AOX [90, 91]. AOX is active *in vivo* but it is difficult to measure what degree of CoQ pool reduction is required for AOX activity. AOX (Figure 2) has been shown to by-pass chemical inhibition of CIII and CIV in human cell culture, flies and mice [86, 90, 91], depletion of CIV subunits in flies [92], mutation of the CIII assembly factor BCS1L [93], increase survival in mice injected with Lipopolysaccharides (LPS) [75], rescue developmental defects caused by interruption of Jun N-terminal Kinase and restore mobility [94] in *dj1β* mutant flies (orthologue of the human PARK7) [90]. However, AOX expression in flies did not rescue mutations in *tko* (fly orthologue of the human MRPS12) [95], *sesB* (orthologue of the Adenine Nucleotide Translocator, ANT 1) [96], *mtDNA-helicase* (orthologue of Twinkle) or *tamas* [97]. Furthermore, AOX lowers survival of mice carrying a muscle-specific mutation of *COX15* [98], aggravates symptoms associated with cardiac arrest, despite preventing ROS-RET, in mouse models where heart circulation is interrupted [99] and severely shortens lifespan of flies under thermal stress, hypoxia and hyperoxia [42].

NDI1 works in the opposite way to AOX, i.e. reduces CoQ to $CoQH_2$ and therefore increases reduction of the CoQ pool [13] (Figure 2). NDI1 from three different species have been expressed in animals, *Aspergillus nidulans* [89], *Saccharomyces cerevisiae* [100] and *Ciona intestinalis [101].* In the fly brain, NDI1 triggers ROS-RET and extends the lifespan of *Drosophila melanogaster* [76, 100, 102]. NDI1 is also able to restore electron transfer to CIII when CI is inhibited. This restores CIII ROS production [103]. Using this strategy, metformin has been shown to protect against tumour growth, reducing CIII ROS by inhibiting CI. When metformin is present CI is blocked and electrons do not reach CIII. Without CIII derived ROS tumours fail to grow, restoring electron flow with NDI1 restores both CIII ROS levels and tumour growth. As with AOX, it is unclear when NDI1 is active, i.e. Is it active constitutively or only when there is an accumulation of NADH in the mitochondrial matrix? In cell culture, NDI1 by-passes both chemical inhibition [103] and genetic depletion of CI [104]. Similarly, NDI1 rescues effects of mutations in several CI subunits in flies [100, 105] and mice [106]. In addition

to extension of *Drosophila* lifespan, NDI1 rescues Parkinson's like *pink1* mutant flies [107], protects against the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone injection in rodent models of Parkinson's disease [108, 109] and reduces damage caused by ischemia-reperfusion in mice [110].

4. **CI and CIII ROS in signalling**

AOX and NDI1 act to induce or supress critical physiological processes via ROS production. This indicates that ROS produced in response to altered redox states of CoQ are instrumental for cellular homeostasis. In the last 20 years, several laboratories have provided evidence that ROS produced specifically at CI (Figure 3) or CIII (Figure 4) control specific physiological processes. This section will discuss a selection of the key events regulated by ROS production at CI or CIII in response to alterations of the $CoQH_2$:CoQ ratio. CI exclusively produces ROS into the mitochondrial matrix, while CIII derived ROS could end up in the matrix or the intermembrane space [82]. This allows compartmentalization of mtROS redox signalling.

4.1 Physiological and pathological importance of CI ROS

In most instances where CI ROS have been shown to be instrumental in the initiation of a specific physiological process, ROS were produced via RET (Figure 3). Until recently RET was considered to be an *in vitro* artefact. As we outlined in the previous section, ROS-RET depends on two parameters which integrate the status of electron flow (redox state of CoQ) and ATP production (pmf). These are the two most vital mitochondrial functions as the remaining mitochondrial roles rely on them. RET results in ROS production at CI that can be modulated both in terms of duration and intensity according to the metabolic status of mitochondria. To confirm that the observed ROS have been produced via RET we need to be able to prevent RET (Figure 3). We can do this by several ways: (i) using rotenone or another inhibitor of the IQ site to block electrons entering CI, (ii) blocking CII to prevent the reduction of CoQ (AOX expression would phenocopy this), or (iii) using uncouplers to dissipate pmf. Reducing the activity of CI or CII using RNAi interference or mutagenesis of subunits will also prevent RET and the use of S1QELs can also help in the validation of ROS-RET. Generally, use of more than one approach to confirm ROS-RET is recommended.

CI linked ROS-RET has been shown to initiate some critical physiological and pathological events. ROS-RET is instrumental *in vitro* for the differentiation of myoblasts into myotubes [111]. It is also the mechanism that induces cell death during an infarct [48]. An infarct has two distinct phases; an ischemic phase when the tissue is deprived of oxygen resulting in the accumulation of succinate, followed by a reperfusion phase, when oxygen is restored to the tissue causing rapid oxidation of the accumulated succinate triggering ROS-RET. Preventing ROS-RET by using rotenone, blocking CII, dissipating pmf or using mitochondrially targeted

antioxidants was sufficient to prevent cell death occurring after reperfusion [48, 112]. This also has implications for organ transplantation. During a transplant, a similar process occurs where an ischemic phase is followed by reperfusion and the production of ROS-RET [113]. Inhibition of CII could help to preserve the integrity of the organ and positively affect transplant outcomes [113].

Respiratory complexes are organised in super structures called supercomplexes [114]. The precise function these supercomplexes [115] is still controversial. They may have evolved to improve the transfer of electrons, reducing electron leak and as a result ROS production [116]. Supercomplexes are dynamic structures that adapt to the various substrates that fuel the ETC. The Enriquez laboratory has described the existence of two distinct pools of CoQ [117]. The respirasome, a supercomplex made by CI+CIII+CIV, utilizes one of these pools, while CII and other dehydrogenases use the other, complementing the respirasome. In mouse cells, the amount of CI and its integration into the respirasome is regulated by ROS-RET [32]. Respiration occurring in conditions where the NADH:$FADH_2$ ratio is high (e.g. glucose oxidation) favour assembly of CI and formation of CI+CIII+CIV supercomplexes. In conditions where the NADH:$FADH_2$ ratio is low (e.g. oxidation of fatty acids), CI is less needed, ROS-RET is triggered and CI is degraded [32]. This degradation reflects an optimization of resources by the ETC.

Cellular adaptation to hypoxia is a classic example of a physiological process regulated by CIII derived ROS. However, oxygen sensing in the blood and cardiorespiratory adaptations triggered by glomus cells of the carotid body in response to low oxygen are due to ROS-RET [74]. Glomus cells have a different metabolism than that of other neurons, dealing with exceptionally high levels of succinate. Hypoxemia increases the oxidation of succinate and elevates the $CoQH_2$:CoQ ratio, subsequently initiating ROS-RET signalling [67]. *Ndufs2* knockout in mice abolishes the hypoxia response of the carotid body without altering the hypercapnia response, demonstrating the specificity of ROS-RET via CI [67, 74]. This response can also be attenuated using antioxidants, confirming the requirement of CI ROS-RET. In mice, CI ROS-RET have been shown to play a pivotal role in pro-inflammatory reprogramming of macrophages in response to bacterial infection [75]. A common way to stimulate the antibacterial response is by exposing cells or animals to LPS. In response to LPS, macrophages undergo metabolic reprogramming, inhibiting oxidative phosphorylation and switching to glycolysis for ATP production. This is essential for the production of pro-inflammatory cytokines such as IL-1β [75]. In line with this, bacterial challenge causes inhibition of CV and boosts succinate oxidation providing conditions which favour ROS-RET. Antioxidants or inhibition of CII with dimethyl-malonate prevents the transcriptomic reprogramming of macrophages as well as production of IL-1β. Furthermore, expression of

AOX protects mice against lethality resulting from LPS injection [75], demonstrating that AOX can supress ROS-RET production in mice *in vivo*.

CI ROS-RET has been shown to regulate longevity in fruit flies. Expression of NDI1 increases the $CoQH_2$:CoQ ratio and ROS in the fly brain [13]. NDI1 flies live longer than controls and have preserved mitochondrial function [76, 100]. ROS-RET production is suppressed in flies fed with rotenone, FCCP or where AOX is co-expressed. AOX co-expression also negated the lifespan extension conferred by NDI1 [13]. Co-expression of NDI1 with a mitochondrially targeted catalase suppressed the positive effect of NDI1 expression on lifespan, while co-expression of SOD2 with NDI1 did not. This indicates that hydrogen peroxide and not superoxide mediates lifespan extension. As we discussed, hydrogen peroxide is the ROS with the best qualities to act as a redox messenger. The Sanz laboratory has shown that ROS-RET is produced in the fly brain without expression of alternative respiratory enzymes. *Drosophila* are usually cultured at 25°C, under these conditions CI produces ROS mainly in the forward direction as shown by the increased ROS levels observed upon rotenone feeding. When exposed to thermal stress either at 29 or 32 °C, in the fly brain, CI switches to RET producing ROS-RET [42], which can be prevented by using rotenone, FCCP, inhibition of CII or co-expression of AOX to block RET. Suppression of ROS-RET with AOX or reduction of hydrogen peroxide levels though expression of a mitochondrially targeted catalase shortens lifespan under thermal, hypoxic and hyperoxic stress. However, overexpression of SOD2, which reduces superoxide but boosts hydrogen peroxide levels, extends lifespan of flies under stress. Mechanistically, suppression of ROS-RET abrogated the transcriptional stress response, indicating that increasing mitochondrial hydrogen peroxide production via ROS-RET is required for stress adaptation. When “anti-stress” ROS signalling is induced in the absence of stress, we observe an extension of lifespan, e.g. with ectopic expression of NDI1 in flies.

Paradoxically, mice expressing AOX are not protected against ischemia-reperfusion and in the long-term accumulate more cellular damage than the controls [99]. Similarly, AOX expression in mice carrying a mutation in *Cox15* shortens the already reduced survival of these mutants [98]. As in wild type flies exposed to stress, reducing mtROS levels in a background of CIV dysfunction prevents many adaptations, including upregulation of autophagy and antioxidant expression. It has been proposed that the increased ROS in *Cox15* mice are generated via RET, but it is also possible that they are generated by CIII (see below). In conclusion, there is significant evidence from a variety of published studies which indicates that low or suppressed levels of mtROS are detrimental under stress and that mitochondrial redox signalling plays a central role in stress adaptation. Therefore, therapies directed to boost

antioxidant defences need to be carefully balanced with the essential role that these mtROS signals play in homeostasis [42].

4.2 Physiological and pathological importance of CIII ROS

CIII in addition to CI is a main source of ROS within the ETC (Figure 4). In the field of ageing, focus on either complex as more critical for ROS production has swung over time [118-120]. Depending on the approach used to measure ROS levels (discussed above) either complex could appear to be the main source of ROS. These caveats can be avoided by performing one more of the following experiments to confirm ROS are produced by CIII *in vivo*: (i) use of an IIIQo specific inhibitor (e.g. myxothiazol), (ii) knockdown or knockout of RISP, (iii) expression of AOX, (iv) inhibition of CI with, e.g. metformin and rescue of the block with NDI1, and (v) use of S3QLs.

IIIQo is the site of ROS production within CIII. ROS produced by CIII are split roughly in half between the matrix and intermembrane space [82]. This allows compartmentalization of ROS production and signal transduction through proteins located in the intermembrane space, and from there to the cytosol. CIII ROS have been proven instrumental for adaptation to hypoxia [121]. Inhibiting the IIIQo site, using myxothiazol or deleting RISP, prevents both CIII ROS generation and the stabilisation of HIF1α [122] [80], which is necessary for cellular adaptation to hypoxia. Similarly, use of S3QELs, that specifically target IIIQo ROS, also prevents stabilisation of HIF1α [123]. Acute hypoxic pulmonary vasoconstriction (AHPV) has also been associated with the generation of mitochondrial CIII ROS, with genetic reduction of CIII inhibiting the cytosolic Ca^{2+} increase required for muscle contraction [124]. AOX mice do not develop a normal AHPV response due to reduced mtROS levels, further supporting a critical role for ROS in this process [125].

CIII ROS are also essential for adaptive immunity. When T cells are stimulated by an antigen, CIII ROS are required for activation of the NFAT (nuclear factor of activated T cells) and subsequent IL-2 production [126]. This is an essential step for complete activation of these immune cells. Antioxidant treatments and suppression of CIII ROS via RISP deletion exclusively in T cells prevented activation but not proliferation, demonstrating that ROS and not ATP is critical for activation. CIII ROS are implicated in tissue repair and cell differentiation. TGF-β is required to initiate organ repair; however, excessive TGF-β signalling results in fibrosis [127]. After binding to its cellular receptor in human lung fibroblasts, TGF-β initiates a transcriptional program that requires CIII ROS [128]. Treatment with mitochondrial antioxidants or genetic depletion of CIII prevent most transcriptional changes associated with this signalling. Excessive fibrosis in patients is associated with abnormal concentrations of mtROS and partially ameliorated by antioxidant treatment. Finally, the Chandel group has

reported that CIII ROS are required for the differentiation of human adipocytes [129]. During the mesenchymal stem cell differentiation, there is an increase in oxygen consumption in parallel to increased mtROS production. Mitochondrial-targeted antioxidants impair the differentiation process, reducing lipid accumulation and expression of PPARγ2 and CEBPα, while addition of external hydrogen peroxide increases the rate of differentiation. The importance of CIII ROS was confirmed by targeting RISP with RNAi, completely blocking differentiation [129].

As with CI ROS, CIII ROS are associated with the onset of specific pathologies. High levels of ROS are found in many cancer types [130], although the exact role that ROS play in the initiation, proliferation and metastasis of tumours is unknown. In K-RAS mediated tumorigenesis, CIII ROS are essential for tumour growth. The anti-cancer effects of the antidiabetic drug metformin could be related to a reduction in CIII ROS [103]. In human cancer cells, metformin inhibits CI and prevents activation of HIF-1α that is essential for the survival of tumours. Patient xenografts fail to grow in mice fed with metformin. Restoring electron flow by expression of NDI1 within the cancer cells abrogated the anti-tumour effects of metformin [103]. These results suggest significant effects of CIII ROS on tumorigenesis and warrant further investigation. For example, it would be interesting to know whether S3QLs could be used to prevent tumour expansion and metastasis. In an independent study the Chandel laboratory has shown that oxidation of $CoQH_2$ to CoQ is required for tumour growth [131]. This was not directly caused by ROS but the requirement of cancer cells to reduce CoQ to $CoQH_2$ for pyrimidine synthesis. Accordingly, AOX expression, which decreases ROS levels, restored tumour growth in cells without CIII [131]. CIII ROS also play a leading role in lung damage induced by contamination [132]. Pollution results in particle accumulation that can damage the lung by inducing mtROS production, which subsequently triggers p53-mediated apoptosis. *In vitro* studies in human cells have shown that IIIQo is the site of ROS generation and that CIII ROS activates both ASK1 and JNK, and through them p53 [132]. In addition to causing cell-death in alveolar epithelial cells, air pollution provokes chronic inflammation in the lung through activation of macrophages [133]. Pollution particles trigger production of CIII ROS in macrophages and the subsequent release of pro-inflammatory cytokine IL-6, responsible for thrombotic episodes in the lung. Metformin reduces the number of electrons reaching CIII, reducing ROS, and protects against thrombosis in mouse models [133]. NDI1 expression in macrophages by-passes metformin inhibition and restores electron flow and IL-6 production. Similarly, S3QELs, but not S1QELs reduced ROS induced by air pollution particles and prevented the generation of IL-6. In a mouse model of chronic obstructive pulmonary disease, AOX expression attenuated damage induced by cigarette smoke [134], indicating that reducing mtROS levels can protect against chronic inflammation in the lung.

4.3 Where are ROS produced when CIV and CV are blocked?

We have reviewed the physiological and pathological processes regulated or induced by ROS produced by either CI or CIII. Despite all the advances we have made in understanding how ROS are produced *in vivo*, we still do not understand how production of ROS by CI or CIII is selectively activated in many physiological settings. For example, which of these two complexes produce ROS when electron flow is interrupted, or proton transfer is reduced? In fact, one important unanswered question in relation to the ETC is where electrons leak from when CIV is blocked. It is possible that ROS are produced from either CIII or CI via RET, and both scenarios can be supported by data from the literature [98, 135]. This is not just an important theoretical query, but highly relevant from a practical point of view, since there are two cellular messengers reported to inhibit CIV *in vivo*. Namely, carbon monoxide (CO) (Zuckerbraun, Chin et al. 2007) and nitric oxide (NO) (Beltran, Quintero et al. 2002) which block CIV and increase mtROS. In addition, CIV activity and/or levels have been shown to decline with age in fly [26], rat [136] and human tissues [30]. This indicates that inhibition of CIV is highly relevant for ageing redox biology.

An age-related decrease in CIV would increase electron leak, contributing to the high levels of ROS described during ageing. The two gaseous messengers, CO & NO, that inhibit CIV reduce CIV activity momentarily, causing an acute increase in mtROS. Once inhibition is relieved, mtROS concentration returns to normal [137]. The capacity to switch a redox signal on and off is a prerequisite of ROS to participating in signalling. This modality of CIV inhibition could be controlled to produce mitochondrial redox signals. For example, an adaptative program reliant on autophagy activation and mitochondrial turnover has been described in mice in response to mutations in CIV subunits [98], such a program is prevented if mtROS signalling is supressed. Physiological increases in the concentration of CO and NO protect against apoptosis during ischemia-reperfusion in mammalian models and extend lifespan in worms [138, 139]. Inhibition of CIV by NO is considered as a method to rapidly regulate mitochondrial respiration [140], activity of CIV is not completely interrupted and a large part of mitochondrial ATP generation is maintained [141]. Although this inhibition is acute, ROS generated as a result initiates long-term adaptions through e.g. relocation of NFkB to the nucleus [141]. Physiological concentrations of CO do not entirely inhibit respiration but shrink oxygen consumption, reducing electron exit and increasing mtROS [142]. While it is unclear where ROS are generated when CO inhibits CIV, a report showing a reduction in ROS following inhibition of CIII in murine macrophages suggests this complex is the source (Zuckerbraun, Chin et al. 2007). CIV inhibition by CO could be a physiological mechanism to protect against oxidative stress. Heme-oxigenase-1 and -2 (HO-1 and HO-2) generate CO as a product of heme degradation. HO-1 is a primary stress response protein and a substantial

increase in HO-1 expression is produced in response to many types of stress, including oxidative stress [143]. It is plausible that CO, that boosts mtROS, is a redox signal that can rise above the noisy background of oxidative stress to activate protective mechanisms. Such a signal could, for example, activate mitochondrial biogenesis to turn over damaged mitochondria [144]. During ageing, mitochondrial respiration is reduced and some reports indicate that this is in part due to the specific decrease in levels and activity of CI and IV [26]. Interruption of electron flow at CIV would cause accumulation of electrons upstream, favour electron leak and ROS production, while CI inhibition would decrease ROS-RET production [13]. It is important to note that this is very different to the controlled and short-lived inhibition of CIV by NO/CO, which causes a reversible increase in ROS versus continually raising ROS causing oxidative stress and interruption of redox signalling. Despite the lack of specific experimental evidence, even just considering the available data, IIIQo looks to be the strongest candidate as a source of electron leak and site of ROS production during the age-associated decrease in mitochondrial respiration [118].

Modulation of CV activity by the endogenous protein ATPase Inhibitory Factor 1 (ATPIF1) alters mtROS production. As with CIV, the source of ROS when CV is inhibited has not yet been determined. CV transports protons from the intermembrane space to the matrix, using the energy generated to produce ATP. Under conditions that compromise electron transport, CV switches and works in reverse to prevent the collapse of pmf [145]. In this mode, expelling protons from the matrix, CV consumes ATP. If CV is in this mode for too long, it can threaten cell survival due to ATP depletion. To prevent cell death ATPIF1 binds CV, preventing the hydrolysis of ATP [146]. The Cuezva laboratory has shown that ATPIF1 can also bind CV when the enzyme is synthesising ATP [147]. In this situation, both Δp and the $CoQH_2$:CoQ ratio are increased since protons cannot return to the matrix and electrons flow slows, increasing the possibility of electron leak [148]. It has been proposed that RET is responsible for boosting ROS when CV is working in reverse, e.g. in response to staurosporine that causes hyperpolarisation of mitochondria [149]. Similar conditions occur when CV is synthesizing ATP and is blocked by oligomycin or the overexpression of ATPIF1. This results in an increase in ROS in both mouse neurons [150] and colonocytes [148, 151]. However, we must be cautious before concluding that ROS are triggered via RET when CV is inhibited, since the role of CI has not been systematically confirmed by genetic or pharmacological inhibition. Like CIV, inhibition of CV by overexpression of ATPIF1 initiates ROS-mediated activation of NFkB [148, 151].

Several manuscripts report lifespan extension as a result of CV inhibition. However, the results are sometimes contradictory. In *Drosophila*, knockdown of *ATPsynD* (the fly orthologue of *ATP5P*) extends lifespan in diets with a low carbohydrate: protein ratio [152]. However,

oligomycin feeding decreases lifespan in both high and low protein diets, while RNAi depletion of CV subunit, *ATPsynβL,* extends lifespan in flies in high protein diets [153]. Inhibition of complex V in flies with J147, a promising new drug in the fight against Alzheimer's, extends lifespan of *Drosophila* [154]. While inhibition of CV increases ROS in flies, the resulting lifespan extension has been associated with modulation of mTOR [152, 154] and not the activation of redox signalling. In *Caenorhabditis elegans*, oligomycin, alpha-ketoglutarate or knock-down of *atp-2*, the worm orthologue of *ATP5F1B*, extends lifespan through inhibition of CV [155]. Under all conditions increased mtROS and downregulation of mTOR signalling was observed. Unfortunately, where and how ROS were produced was not investigated. It has been reported recently that alpha-ketoglutarate supplementation in mice extends lifespan [156]. In this study, no alterations in mTOR signalling were found, but alpha-ketoglutarate strongly reduced inflammation, suggesting a possible role for mtROS in the lifespan extension, through regulation of the inflammatory response [157] .

5. The problem with mitochondrial redox signalling during ageing

We know that mitochondrial redox signalling is essential for cellular homeostasis, that ROS produced either by CI or CIII can regulate crucial physiological processes, and that excessive levels of ROS production can contribute to tissue damage and the onset of many pathologies. During ageing an accumulation of defective mitochondria generate excessive amounts of ROS. This not only provokes oxidative stress but interferes with mitochondrial redox signalling and the many processes it regulates.

5.1 Are mtROS produced differently in old cells?

One clear distinction between "young" and "old" mitochondria is the relative amount of ROS production, which is markedly increased in aged mitochondria. This phenomenon has been observed in worms, flies, rodents and humans in various tissues and cell types [158-161]. It is unclear if ROS found in aged tissues are of the same nature found in young tissues and whether they derive from the same or a different set of ROS generators. We should clarify that there is no consistent change in either the levels or efficiency of antioxidants in old individuals [3]. This means we can discount a failure of antioxidant systems as an explanation for this age associated increase in ROS. If we look to the ETC there are several possible explanations for the higher electron leak observed in aged mitochondria: (i) potential ROS generators that are inactive in young cells may become active, (ii) there maybe constitutive activation of redox switches which produce ROS in response to a physiological signal (iii) ROS are simply being produced at higher levels and finally (iv) the nature of ROS being produced could be altered with a shift from loROS to hiROS, or an increase in hiROS.

There are currently very few studies dedicated to dissecting in detail how mitochondria from old individuals produce ROS. There is *in vitro* data which indicate that old mitochondria produce ROS in fundamentally the same way to young mitochondria, i.e. CI and CIII being the main generators [119, 162-164]. These studies show that during ageing, the amount of ATP generated per molecule of oxygen consumed is reduced. In parallel, to a reduction in OXPHOS efficiency, there is an increase in the amount of ROS production in both postmitotic as well as senescent cells [13, 162, 165], indicating that the ETC is leakier in old mitochondria and that ROS production is less controlled during ageing. In senescent cells, there is an increase in ROS per mitochondrion as well as an increase in the number of mitochondria [166]. This rise in ROS observed during ageing is linear in worms and fruit flies [13, 158, 159]. Despite the lack of conclusive experimental evidence, we can speculate that an initial increase in mtROS could be the result of adaptive redox signalling, with mitochondria attempting to activate mechanism(s) to maintain cellular homeostasis. However, the increase observed at older ages is probably the result of unrestricted ROS production, which leads to the accumulation of oxidative damage [167]. Accumulation of oxidized proteins, lipids, and DNA in the brain is more conspicuous in older vs middle-aged individuals [30] and is a hallmark of neurodegenerative diseases such as Parkinson's or Alzheimer's [168, 169]. This suggests that oxidative damage is most likely due to an accumulation of hiROS, generated through the reaction of loROS with free metals, and not through the direct generation of hiROS in the ETC.

5.2 How is mtROS production affected when electron flow is reduced during ageing?

As we discussed in a previous section, reduction in CIV activity by NO or CO causes an increase in the redox state of CoQ pool contributing to electron leak at CI and CIII. Ageing is characterised by the accumulation of mitochondria, which respire less and have a lower pmf [170]. Reduction in the levels and/or activity of CIV during ageing has been described both in insects and mammals [26, 30, 136]. The difference between the age-linked reduction in CIV and an acute inhibition by CO/NO is that the former results in a constant increase in the levels of mtROS. Age-linked reduction in CIV function could elicit the generation of ROS by CIII, since electrons will circulate slowly, the half-life of $CoQ^{\cdot}$ within CIII will increase as well as electron leak and ROS production. The chronic increase in ROS caused by age-related CIV dysfunction will disrupt CIII redox signalling, affecting many important processes as discussed earlier in this review. ROS measurements in isolated mitochondria from rat hearts support this model [171]. However, we still need further confirmation of this in mitochondria from other tissues and in other species as well as *in vivo* validation.

The age-associated reduction in electron flow decreases the capacity of mitochondria to generate a pmf high enough to produce a ROS-RET signal. As we have discussed, preventing

ROS-RET in flies reduces their capacity to adapt to and survive stress [42]. It is possible that in very old individuals, mitochondria cannot initiate ROS-RET signalling, preventing stress adaptation and reducing survival. This is further aggravated by the age-related reductions in CI levels observed in several animal species [13, 30], which would reduce the intensity of the signal. Another indicator that ROS-RET is probably diminished during ageing is the reduction in the $CoQH_2$:CoQ ratio detected in specialized studies. No decrease in the levels of $CoQH_2$ and CoQ has been reported in several mouse tissues [172] or human plasma [173]. However, a rise in CoQ levels (with a decrease in $CoQH_2$) during ageing has been reported in three animal species i.e. worms, pigs and mice [174]. In addition, $CoQH_2$ levels positively correlate with muscle strength in humans [175], indicating that sarcopenia (an important hallmark of ageing) is associated with more CoQ. However, we must be cautious when interpreting these studies as they report total, and not mitochondrial, levels of CoQ and $CoQH_2$, so may not reflect the situation within mitochondria. In fact, the authors, related their findings to an increase in oxidative stress, as $CoQH_2$ is a powerful antioxidant, and not with problems in mitochondrial function. In future, it would be important to determine the nature of changes in mitochondrial levels of $CoQH_2$ and CoQ during ageing, particularly considering the likely existence of two separate pools [116]. In summary, the age-related depression in mitochondrial respiration would affect CoQ signalling causing uninterrupted electron leak at IIIQo and making the generation of a ROS-RET extremely difficult and negatively affecting the processes regulated by this signal.

5.3. High levels of ROS interrupt redox signalling during ageing

High levels of ROS cause damage by either provoking oxidative stress and/or interrupting redox signalling. The latter occurs when downstream targets of ROS signalling stop responding to changes in the redox environment. For example, peroxiredoxins which have been shown to be involved in transduction of redox signals are inactivated by hyper oxidation [176]. Given the chronically high levels of ROS in aged individuals, it is likely that many redox targets would be in this permanently oxidized state and unable to transduce signals produced in response to changes in ROS levels. Indeed, increases in oxidized forms of both NADPH and glutathione have been observed in whole fly homogenates and several rodent tissues [177-179]. The group of John Tower has provided evidence that the transcriptome of *Drosophila melanogaster* adapts to the high levels of oxidative stress observed during ageing. In a seminal study [180], they compared the transcriptome of old flies with that of young flies exposed to four types of stress; heat, hydrogen peroxide, hyperoxia and ionising radiation. The transcriptomic changes seen in aged flies were most similar to those seen as a result of hyperoxia. This is in line with the oxidative stress caused by hyperoxia which might approximate the levels of oxidative stress in aged flies.

Several studies indicate that mitochondrial redox signalling is able to be extremely precise [181]. One of the mechanisms by which such precision is achieved is reversible oxidation of cysteine residues (Reczek and Chandel 2014). The laboratories of Stefan Drose and Ulrich Brandt have shown that cysteine oxidation of specific proteins occurs exclusively in response to ROS production by CI or CIII [182]. In their study, they found that certain proteins were oxidized by CIII ROS (e.g. TIM50 or carnitine O-acetyltransferase), while others were oxidised by CI ROS (e.g. dihydrolipoyl dehydrogenase or succinate dehydrogenase flavoprotein). This type of precision is only possible in a context free of 'noisy' non-specific ROS resulting in non-selective protein oxidation, i.e. in young individuals. In fruit flies, fasting elicits a particular redox response that alters the level of oxidation or reduction of cysteines within proteins dedicated to stress adaptation [183]. This is particularly relevant when you consider fasting has also been reported to induce production of ROS-RET in isolated mitochondria from rat livers [184]. In flies, however, neither hydrogen peroxide nor paraquat resulted in specific redox responses, and oxidation of cysteine residues was not increased in aged flies. Similar results have also been reported for various tissues collected from young and old mice [185]. The laboratory of Edward Chouchani had recently published to date the most complete redoxome map in an animal. They did not find any consistent increase in the number of oxidized cysteines as a result of ageing [185]. However, they found disorganization of redox networks established in young animals, i.e. proteins that respond to changes in ROS levels in a coordinated way. This fits with the observation where the response to ROS in old individuals is perturbed. Proteins most affected by this perturbation were those involved in the stress response in young individuals, while cysteine residues that were not altered in young individuals became hyper-reactive. Finally, many proteins found to be more oxidized in old mice have been reported to be involved in the onset of many age-related diseases in humans [185].

In summary, existing data indicate that as well as causing oxidative stress, high levels of ROS interrupt redox signalling. We have seen that $CoQH_2$/CoQ redox signalling controls essential processes and age-linked disruption in redox signalling will undoubtedly affect them. Dysregulation of redox signalling may explain, why cancer and cellular senescence increase with age, why stress is more difficult to cope with, why many infections are more challenging after a certain age and why when we are old we struggle to recover from a heart attack or stroke.

5.4 Why do damaged mitochondria accumulate during ageing?

It is clear that an accumulation of damaged mitochondria, generating high levels of mtROS is likely a significant contributor to the onset of age-related diseases. Therefore, the substitution of these damaged organelles with "youthful" ones would be an ideal anti-ageing strategy. To

prevent, delay or reverse this build-up of defective mitochondria, we need to understand how and why it happens. Four different mechanisms have been postulated to explain why this might be occurring [186]: (i) the vicious circle, (ii) ROS induced ROS, (iii) failure in quality control, and (iv) the epigenetic mechanism. The vicious circle hypothesis proposes that mtROS cause mutations in mtDNA, which increase mtROS that cause further mutations. Although logical, this hypothesis has been debunked by two experimental models. Firstly, the induction of mutations in mtDNA through knock-in of proof reading deficient mitochondrial polymerase gamma does not increase mtROS levels in mice [187]. Secondly, mutations of *Mclk1* (that participates in CoQ biosynthesis) causes an interruption in electron flow and increases in mtROS levels and reduces lifespan [188]. The interruption of CoQ biosynthesis is reversible through the administration of 2,4-dihydroxybenzoic acid which restores mitochondrial respiration, reduces mtROS levels and rescues lifespan. The rescue in lifespan occurs even when mice are treated after one year of high levels of ROS. In addition, experimentally boosting mtROS levels in flies extends lifespan without decreasing mitochondrial respiration [13]. These data indicate that extremely high levels of mtROS during a significant period of lifespan in mice or flies does not have a negative impact on long-term survival. We can conclude that there is no ROS "memory", which contradicts both the idea that ROS cause ageing and the vicious circle hypothesis.

An alternative explanation for the age associated increase in mtROS relates to the observation that ROS production in one cellular location triggers ROS production elsewhere [186]. For example, there is tight coordination between NOXs and mitochondria to produce ROS [189]. In the context of ageing, an increase from one of two ROS sources causes an increase from the other, initiating a feedback loop leading to mitochondria producing higher and higher amounts of ROS, attempting to communicate information for maintaining metabolic homeostasis. ROS producers which are dormant in young, but active in old individuals may also be involved in driving mitochondria to produce more ROS in order to produce a signal that can be detected above "noisy" ROS, i.e. ROS not involved in redox signalling. Possible extra-mitochondrial sources of ROS include both NOS [190] and xanthine oxidase (XO) [191]. NOS and XO begin producing superoxide and hydrogen peroxide in the presence of high concentrations of oxidants [192]. The ROS they produce attack mitochondria causing the formation of more mtROS [193]. XO [194] and NOS [195] have both been shown to produce more ROS in various aged mouse tissues. Although further confirmation is required there is sufficient evidence to support that a "ROS induced ROS" mechanism is partially responsible for increased mtROS during ageing. However, two questions remain unanswered. Firstly, is increased mtROS an attempt to increase the intensity of a ROS signal or a result of oxidative damage? Secondly, what causes the initiation of this ROS feedback loop which progressively

increases more and more mtROS levels during ageing? The decline in quality control mechanisms that turnover damaged mitochondria during ageing [196, 197] has been proposed to underlie this feedback loop. A quality control failure would explain why defective mitochondria accumulate and boosting mechanisms of quality control, such as autophagy or the proteasome, increases lifespan across different animal species [198-200]. Furthermore, specifically targeting mitophagy extends lifespan and preserves mitochondrial function both in worms and flies [201-203]. Extended maintenance of mitochondrial function delays the age-linked increased in mtROS [202]. All in all, these results suggest that maintaining healthy mitochondria for longer facilitates a healthier lifespan. Since many mechanisms of quality control are redox-regulated, the age-related increase in oxidative stress could contribute to their decline [204, 205]. For example, SQSTM1/p62 is one of the best characterized autophagic receptors, instrumental in recycling ubiquitylated proteins and organelles. In mice and humans, oxidation of two specific cysteine residues causes p62 oligomerisation, increasing its capacity to remove damaged proteins [206]. Mutations of p62 that disrupt these redox-sensitive cysteines have been associated with neurodegeneration in humans [207]. Conversely, expression of a humanized Ref(2)p (fly orthologue of p62) by introduction of these redox-sensitive cysteine residues increases the resistance of flies to oxidative and heat stress [206]. This redox-sensitive mechanism supports oligomerization of p62 and increases recycling capacity in young, healthy individuals, where mtROS are kept low. In old individuals characterized by mitochondria producing high levels of mtROS, it can promote the aggregation and precipitation of p62 (and associated proteins), which has been observed in old mice and rats [206, 208] as well as in human neurodegenerative diseases [209]. Saturation of the lysosomal system has recently been proposed as the mechanism underlying accumulation of defective mitochondria in the context of mitochondrial disease [210]. It is not difficult to imagine that something similar (i.e. saturation of the lysosomal system) may happen in the context of ageing [211], which will worsen the accumulation of defective mitochondria.

If reduced quality control allows the accumulation of defective mitochondria, we must understand why quality control fails during ageing. There are two explanations that complement each other. The first is that there is an irreversible accumulation of damaged molecules both within and outside of the mitochondria that overwhelm quality control. The former hypothesis is widely accepted and is supported by the accumulation of macromolecular damage during ageing in lipid, proteins, and DNA across different species [212, 213]. The second explanation is that genetic or epigenetic changes negatively affect quality control and subsequently increase dysfunctional mitochondria. Indeed, there is a strong connection between mitochondrial function and epigenome reprogramming. As we have seen, mitochondrial activation is required for the differentiation of both myoblasts and adipocytes

[111, 129]. Human neurons also require an energetic switch from glycolysis to oxidative phosphorylation for differentiation [214]. Conversely reprogramming embryonic fibroblasts into pluripotent stem cells requires shifting from oxidative phosphorylation to glycolysis [215]. Epigenomic dysregulation is starting to be considered as a primary driver of ageing [216, 217]. Accordingly, epigenetic clocks are among the best markers of biological age [218]. Age-linked epigenetic changes can affect mitochondrial function, reducing quality control and/or alter super-complex organization making the ETC leakier, producing "noisy" ROS [202, 219]. Specific epigenetic changes occurring during ageing such as the reduction in H3K9me3 [220] can impact the expression of mitochondrial genes [221], causing a characteristic reduction in mitochondrial gene expression noted in multiple ageing studies [222]. Epigenetic alterations can also cause metabolic alterations that impact mitochondria. For example, both sirtuins and poly-ADP ribose polymerases are essential to maintain genomic and epigenetic stability, they use NAD+ as a co-factor; therefore, an age-linked increase in genome instability that boosts the activity of these enzymes can cause depletion of NAD+. Accordingly, it has been shown that age-related decrease in NAD+ causes the accumulation of defective mitochondria in mice while boosting NAD+ levels in old mice restores mitochondrial function [223]. Treating mouse models of progeria (caused by mutations in enzymes involved in maintaining genome stability) with NAD+ precursors rescued nuclear defects as well as mitochondrial deterioration [224]. Similar results have been reproduced in worms, flies and patient cells [225] pointing to enhancement of NAD+ metabolism as a promising anti-ageing treatment.

5.5 Strategies to preserve redox signalling during ageing.

Finally, we will discuss the currently available strategies to maintain redox signalling during ageing. Strategies that delay ageing such as dietary restriction, intermittent fasting, reduced insulin/insulin-like signalling and repression of TOR signalling also retard the accumulation of defective mitochondria [226]. These approaches, at best, delay the appearance of dysfunctional organelles, but do not prevent accumulation completely and result in similar problems later in life. It would be interesting to identify ways of preserving or restoring mitochondrial redox signalling, eliminating or reducing the pervasive effects associated with sustained high levels of mtROS, e.g. chronic inflammation [227], senescence [165] or cancer [103].

One of the most exciting approaches to improve mitochondrial function is to target epigenomic changes or the metabolic consequences of these changes. Genetic manipulation of enzymes involved in epigenetic regulation extends lifespan in flies and worms. For example, in flies, overexpression of repressors of transposable elements such as Su(var)3-9 or Dicer-2 extends lifespan [228], whereas in worms knock-down of certain demethylases, like UTX-1, also

increases longevity [229]. Genetic manipulation or even modifying the epigenome with drugs can be both challenging and dangerous. A more practical strategy may be to compensate for the adverse metabolic consequences of those epigenetic changes that affect mitochondrial function. We have argued that depletion of NAD+ causes the accumulation of defective mitochondria and stimulating NAD+ synthesis restores mitochondrial function in old mice [223]. An essential part of the effect of NAD+ is attributed to enhancing mitophagy. Similarly inducing mitophagy, by feeding worms with Urolithin A, extends lifespan and increases muscle strength in old mice and rats [201]. Alternatively, inducing macro autophagy can be more straightforward and produce a similar outcome [230]. Rapamycin and spermidine are well-characterized inductors of autophagy that improve mitochondrial respiration by removal of defective mitochondria [230, 231]. We will need to wait to see a systematic analysis of the effects of NAD+ or other mitophagy boosters in clinical trials to decide if they are useful or not in humans.

A more targeted solution would be the use of alternative respiratory enzymes or antioxidants. Alternative enzymes can help to normalize CoQ-mediated redox signalling. Both AOX and NDI1 have been shown to be protective in several disease models (see above), but only NDI1 extends lifespan and protects mitochondrial function during ageing [13]. AOX has negative effects on flies reducing stress resilience [42]. Alternative respiratory enzymes are beneficial for research, but it is difficult to envision their use in the clinic for both technical and ethical reasons. The efficacy of antioxidants has been shown to be disappointing without any apparent effects on lifespan and mixed results regarding mitochondrial function [3]. There are some exceptions to the rule and evidence of positive effects on lifespan such as expression of a mitochondrially targeted catalase in mice [232], or overexpression of peroxiredoxin 5 in fruit flies [233]. However, expression of a mitochondrially targeted catalase has been shown to negatively impact both brain metabolism and behaviour in mice [43]. On the other hand, peroxiredoxins may extend lifespan not by protecting against oxidative stress but by increasing redox signalling. For example, peroxiredoxin 2 is required in *Caenorhabditis elegans* for the lifespan extension conferred by metformin feeding [234]. Metformin stimulates mitochondrial redox signalling in worms, and without peroxiredoxin 2 the pro-survival pathways controlled by SKN-1 are not activated. In future, it would be interesting to test whether peroxiredoxins can help to preserve mitochondrial function and extend lifespan by supporting mitochondrial redox signalling in mice. A new generation of antioxidants that can improve signalling in the future are S1QELs and S3QELs. S1QELs can be administered after a heart attack, while S3QELs can help to prevent the excess of CIII ROS that may occur during ageing due to a decrease in electron flow (see above). However, S1/3QELs have not been widely used in animal or cell culture models, or proven clinically effective yet so we need to know more how

they operate *in vivo* and whether they present some side effects. Finally, one solution to improve redox signalling during ageing may be trying to avoid triggering oxidative stress by sequestering free iron. Iron accumulates during ageing in flies [235], several mouse organs [236] and human muscle [237]. Furthermore, mitochondria are particularly prone to iron accumulation [238]. Mitochondrial iron accumulation combined with high levels of mtROS produces hiROS and oxidative damage. In *Drosophila,* reducing age-linked iron accumulation extends lifespan [239, 240]. Preventing the accumulation of iron deposits or chelating free iron during ageing could be a strategy to reduce oxidative damage, but also to enhance redox signalling.

6. Conclusions.

In this review, we have seen the importance of mtROS signalling in deciding cell fate and cellular homeostasis. CI and CIII are the two main generators of ROS within mitochondria according to both *in vitro* and *in vivo* evidence. ROS produced at CI or CIII control specific physiological processes ranging from cell differentiation to oxygen sensing. Similarly, ROS production by both CI and CIII triggers pathological processes such as ischemia-reperfusion and cancer. The $CoQH_2$:CoQ ratio is a significant determinant of electron leak and ROS production in the ETC. Producing ROS in response to changes in the redox state of the CoQ pool is an ideal way to signal metabolic status activating a mitochondrial response that coordinates the cellular response to external and internal changes. During ageing, redox signalling is altered by the accumulation of defective mitochondria. It is unknown why damaged mitochondria accumulate, but epigenetic alterations that deplete NAD+ levels could be the ultimate cause. Accordingly, novel therapies targeted to prevent or correct the consequences of epigenomic dysregulation are the most likely to restore redox signalling an help us to live healthier for longer.

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7. References.

[1] C. Lopez-Otin, M.A. Blasco, L. Partridge, M. Serrano, G. Kroemer, The hallmarks of aging, Cell 153(6) (2013) 1194-217.
[2] D.C. Wallace, A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine, Annu Rev Genet 39 (2005) 359-407.
[3] A. Sanz, R. Pamplona, G. Barja, Is the mitochondrial free radical theory of aging intact?, Antioxid Redox Signal 8(3-4) (2006) 582-99.
[4] G. Barja, Free radicals and aging, Trends Neurosci 27(10) (2004) 595-600.
[5] D. Gems, R. Doonan, Antioxidant defense and aging in C. elegans: is the oxidative damage theory of aging wrong?, Cell Cycle 8(11) (2009) 1681-7.
[6] A. Sanz, R.K. Stefanatos, The mitochondrial free radical theory of aging: a critical view, Curr Aging Sci 1(1) (2008) 10-21.
[7] S. Hekimi, J. Lapointe, Y. Wen, Taking a "good" look at free radicals in the aging process, Trends Cell Biol 21(10) (2011) 569-76.
[8] A. Sanz, Mitochondrial reactive oxygen species: Do they extend or shorten animal lifespan?, Biochim Biophys Acta (2016).
[9] P. Ghezzi, A.D. Mooradian, Demystifying Oxidative Stress, Handb Exp Pharmacol (2020).
[10] E. Owusu-Ansah, U. Banerjee, Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation, Nature 461(7263) (2009) 537-41.
[11] M. Ristow, K. Zarse, A. Oberbach, N. Kloting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Bluher, Antioxidants prevent health-promoting effects of physical exercise in humans, Proc Natl Acad Sci U S A 106(21) (2009) 8665-70.
[12] R. Zhou, A.S. Yazdi, P. Menu, J. Tschopp, A role for mitochondria in NLRP3 inflammasome activation, Nature 469(7329) (2011) 221-5.
[13] F. Scialo, A. Sriram, D. Fernandez-Ayala, N. Gubina, M. Lohmus, G. Nelson, A. Logan, H.M. Cooper, P. Navas, J.A. Enriquez, M.P. Murphy, A. Sanz, Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan, Cell Metab 23(4) (2016) 725-34.
[14] F. Scialo, D.J. Fernandez-Ayala, A. Sanz, Role of Mitochondrial Reverse Electron Transport in ROS Signaling: Potential Roles in Health and Disease, Front Physiol 8 (2017) 428.
[15] W. Droge, Free radicals in the physiological control of cell function, Physiol Rev 82(1) (2002) 47-95.
[16] J. Egea, I. Fabregat, Y.M. Frapart, P. Ghezzi, A. Gorlach, T. Kietzmann, K. Kubaichuk, U.G. Knaus, M.G. Lopez, G. Olaso-Gonzalez, A. Petry, R. Schulz, J. Vina, P. Winyard, K. Abbas, O.S. Ademowo, C.B. Afonso, I. Andreadou, H. Antelmann, F. Antunes, M. Aslan, M.M. Bachschmid, R.M. Barbosa, V. Belousov, C. Berndt, D. Bernlohr, E. Bertran, A. Bindoli, S.P. Bottari, P.M. Brito, G. Carrara, A.I. Casas, A. Chatzi, N. Chondrogianni, M. Conrad, M.S. Cooke, J.G. Costa, A. Cuadrado, P. My-Chan Dang, B. De Smet, B. Debelec-Butuner, I.H.K. Dias, J.D. Dunn, A.J. Edson, M. El Assar, J. El-Benna, P. Ferdinandy, A.S. Fernandes, K.E. Fladmark, U. Forstermann, R. Giniatullin, Z. Giricz, A. Gorbe, H. Griffiths, V. Hampl, A. Hanf, J. Herget, P. Hernansanz-Agustin, M. Hillion, J. Huang, S. Ilikay, P. Jansen-Durr, V. Jaquet, J.A. Joles, B. Kalyanaraman, D. Kaminskyy, M. Karbaschi, M. Kleanthous, L.O. Klotz, B. Korac, K.S. Korkmaz, R. Koziel, D. Kracun, K.H. Krause, V. Kren, T. Krieg, J. Laranjinha, A. Lazou, H. Li, A. Martinez-Ruiz, R. Matsui, G.J. McBean, S.P. Meredith, J. Messens, V. Miguel, Y. Mikhed, I. Milisav, L. Milkovic, A. Miranda-Vizuete, M. Mojovic, M.

Monsalve, P.A. Mouthuy, J. Mulvey, T. Munzel, V. Muzykantov, I.T.N. Nguyen, M. Oelze, N.G. Oliveira, C.M. Palmeira, N. Papaevgeniou, A. Pavicevic, B. Pedre, F. Peyrot, M. Phylactides, G.G. Pircalabioru, A.R. Pitt, H.E. Poulsen, I. Prieto, M.P. Rigobello, N. Robledinos-Anton, L. Rodriguez-Manas, A.P. Rolo, F. Rousset, T. Ruskovska, N. Saraiva, S. Sasson, K. Schroder, K. Semen, T. Seredenina, A. Shakirzyanova, G.L. Smith, T. Soldati, B.C. Sousa, C.M. Spickett, A. Stancic, M.J. Stasia, H. Steinbrenner, V. Stepanic, S. Steven, K. Tokatlidis, E. Tuncay, B. Turan, F. Ursini, J. Vacek, O. Vajnerova, K. Valentova, F. Van Breusegem, L. Varisli, E.A. Veal, A.S. Yalcin, O. Yelisyeyeva, N. Zarkovic, M. Zatloukalova, J. Zielonka, R.M. Touyz, A. Papapetropoulos, T. Grune, S. Lamas, H. Schmidt, F. Di Lisa, A. Daiber, European contribution to the study of ROS: A summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS), Redox biology 13 (2017) 94-162.
[17] M.D. Brand, Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling, Free Radic Biol Med 100 (2016) 14-31.
[18] M. Bajzikova, J. Kovarova, A.R. Coelho, S. Boukalova, S. Oh, K. Rohlenova, D. Svec, S. Hubackova, B. Endaya, K. Judasova, A. Bezawork-Geleta, K. Kluckova, L. Chatre, R. Zobalova, A. Novakova, K. Vanova, Z. Ezrova, G.J. Maghzal, S. Magalhaes Novais, M. Olsinova, L. Krobova, Y.J. An, E. Davidova, Z. Nahacka, M. Sobol, T. Cunha-Oliveira, C. Sandoval-Acuna, H. Strnad, T. Zhang, T. Huynh, T.L. Serafim, P. Hozak, V.A. Sardao, W.J.H. Koopman, M. Ricchetti, P.J. Oliveira, F. Kolar, M. Kubista, J. Truksa, K. Dvorakova-Hortova, K. Pacak, R. Gurlich, R. Stocker, Y. Zhou, M.V. Berridge, S. Park, L. Dong, J. Rohlena, J. Neuzil, Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells, Cell Metab 29(2) (2019) 399-416 e10.
[19] I. Martinez-Reyes, L.P. Diebold, H. Kong, M. Schieber, H. Huang, C.T. Hensley, M.M. Mehta, T. Wang, J.H. Santos, R. Woychik, E. Dufour, J.N. Spelbrink, S.E. Weinberg, Y. Zhao, R.J. DeBerardinis, N.S. Chandel, TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions, Mol Cell 61(2) (2016) 199-209.
[20] A. Logan, V.R. Pell, K.J. Shaffer, C. Evans, N.J. Stanley, E.L. Robb, T.A. Prime, E.T. Chouchani, H.M. Cocheme, I.M. Fearnley, S. Vidoni, A.M. James, C.M. Porteous, L. Partridge, T. Krieg, R.A. Smith, M.P. Murphy, Assessing the Mitochondrial Membrane Potential in Cells and In Vivo using Targeted Click Chemistry and Mass Spectrometry, Cell Metab 23(2) (2016) 379-85.
[21] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem J 417(1) (2009) 1-13.
[22] D. Harman, The free radical theory of aging, Antioxid Redox Signal 5(5) (2003) 557-61.
[23] H. Sies, D.P. Jones, Reactive oxygen species (ROS) as pleiotropic physiological signalling agents, Nat Rev Mol Cell Biol 21(7) (2020) 363-383.
[24] S. Goodell, G. Cortopassi, Analysis of oxygen consumption and mitochondrial permeability with age in mice, Mech Ageing Dev 101(3) (1998) 245-56.
[25] K. Takahashi, M. Takahashi, Exogenous administration of coenzyme Q10 restores mitochondrial oxygen consumption in the aged mouse brain, Mech Ageing Dev 134(11-12) (2013) 580-6.
[26] M. Ferguson, R.J. Mockett, Y. Shen, W.C. Orr, R.S. Sohal, Age-associated decline in mitochondrial respiration and electron transport in Drosophila melanogaster, Biochem J 390(Pt 2) (2005) 501-11.
[27] K. Yasuda, T. Ishii, H. Suda, A. Akatsuka, P.S. Hartman, S. Goto, M. Miyazawa, N. Ishii, Age-related changes of mitochondrial structure and function in Caenorhabditis elegans, Mech Ageing Dev 127(10) (2006) 763-70.

[28] K.F. Petersen, D. Befroy, S. Dufour, J. Dziura, C. Ariyan, D.L. Rothman, L. DiPietro, G.W. Cline, G.I. Shulman, Mitochondrial dysfunction in the elderly: possible role in insulin resistance, Science 300(5622) (2003) 1140-2.
[29] F. Scialo, V. Mallikarjun, R. Stefanatos, A. Sanz, Regulation of lifespan by the mitochondrial electron transport chain: reactive oxygen species-dependent and reactive oxygen species-independent mechanisms, Antioxid Redox Signal 19(16) (2013) 1953-69.
[30] R. Cabre, A. Naudi, M. Dominguez-Gonzalez, V. Ayala, M. Jove, N. Mota-Martorell, G. Pinol-Ripoll, M.P. Gil-Villar, M. Rue, M. Portero-Otin, I. Ferrer, R. Pamplona, Sixty years old is the breakpoint of human frontal cortex aging, Free Radic Biol Med 103 (2017) 14-22.
[31] S. Guerrero-Castillo, F. Baertling, D. Kownatzki, H.J. Wessels, S. Arnold, U. Brandt, L. Nijtmans, The Assembly Pathway of Mitochondrial Respiratory Chain Complex I, Cell Metab 25(1) (2017) 128-139.
[32] A. Guaras, E. Perales-Clemente, E. Calvo, R. Acin-Perez, M. Loureiro-Lopez, C. Pujol, I. Martinez-Carrascoso, E. Nunez, F. Garcia-Marques, M.A. Rodriguez-Hernandez, A. Cortes, F. Diaz, A. Perez-Martos, C.T. Moraes, P. Fernandez-Silva, A. Trifunovic, P. Navas, J. Vazquez, J.A. Enriquez, The CoQH2/CoQ Ratio Serves as a Sensor of Respiratory Chain Efficiency, Cell reports 15(1) (2016) 197-209.
[33] E.S. Vincow, G. Merrihew, R.E. Thomas, N.J. Shulman, R.P. Beyer, M.J. MacCoss, L.J. Pallanck, The PINK1-Parkin pathway promotes both mitophagy and selective respiratory chain turnover in vivo, Proc Natl Acad Sci U S A 110(16) (2013) 6400-5.
[34] M. Hansen, D.C. Rubinsztein, D.W. Walker, Autophagy as a promoter of longevity: insights from model organisms, Nat Rev Mol Cell Biol 19(9) (2018) 579-593.
[35] Y. Wang, R. Branicky, A. Noe, S. Hekimi, Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling, J Cell Biol 217(6) (2018) 1915-1928.
[36] J.D. Brown, A.M. Day, S.R. Taylor, L.E. Tomalin, B.A. Morgan, E.A. Veal, A peroxiredoxin promotes H2O2 signaling and oxidative stress resistance by oxidizing a thioredoxin family protein, Cell reports 5(5) (2013) 1425-35.
[37] M.C. Sobotta, W. Liou, S. Stocker, D. Talwar, M. Oehler, T. Ruppert, A.N. Scharf, T.P. Dick, Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling, Nat Chem Biol 11(1) (2015) 64-70.
[38] E.A. Veal, Z.E. Underwood, L.E. Tomalin, B.A. Morgan, C.S. Pillay, Hyperoxidation of Peroxiredoxins: Gain or Loss of Function?, Antioxid Redox Signal 28(7) (2018) 574-590.
[39] G. Esposito, M. Vos, S. Vilain, J. Swerts, J. De Sousa Valadas, S. Van Meensel, O. Schaap, P. Verstreken, Aconitase causes iron toxicity in Drosophila pink1 mutants, PLoS Genet 9(4) (2013) e1003478.
[40] M.A. Smith, P.L. Richey Harris, L.M. Sayre, J.S. Beckman, G. Perry, Widespread peroxynitrite-mediated damage in Alzheimer's disease, J Neurosci 17(8) (1997) 2653-7.
[41] S.G. Rhee, Cell signaling. H2O2, a necessary evil for cell signaling, Science 312(5782) (2006) 1882-3.
[42] F. Scialo, A. Sriram, R. Stefanatos, R.V. Spriggs, S.H.Y. Loh, L.M. Martins, A. Sanz, Mitochondrial complex I derived ROS regulate stress adaptation in Drosophila melanogaster, Redox biology 32 (2020) 101450.
[43] C. Vicente-Gutierrez, N. Bonora, V. Bobo-Jimenez, D. Jimenez-Blasco, I. Lopez-Fabuel, E. Fernandez, C. Josephine, G. Bonvento, J.A. Enriquez, A. Almeida, J.P. Bolanos, Astrocytic mitochondrial ROS modulate brain metabolism and mouse behaviour, Nat Metab 1(2) (2019) 201-211.
[44] M. Baumgart, S. Priebe, M. Groth, N. Hartmann, U. Menzel, L. Pandolfini, P. Koch, M. Felder, M. Ristow, C. Englert, R. Guthke, M. Platzer, A. Cellerino, Longitudinal RNA-Seq

Analysis of Vertebrate Aging Identifies Mitochondrial Complex I as a Small-Molecule-Sensitive Modifier of Lifespan, Cell Syst 2(2) (2016) 122-32.
[45] M. Ristow, K. Zarse, How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis), Exp Gerontol 45(6) (2010) 410-8.
[46] W. Yang, S. Hekimi, A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans, PLoS Biol 8(12) (2010) e1000556.
[47] E. Owusu-Ansah, W. Song, N. Perrimon, Muscle mitohormesis promotes longevity via systemic repression of insulin signaling, Cell 155(3) (2013) 699-712.
[48] E.T. Chouchani, V.R. Pell, E. Gaude, D. Aksentijevic, S.Y. Sundier, E.L. Robb, A. Logan, S.M. Nadtochiy, E.N. Ord, A.C. Smith, F. Eyassu, R. Shirley, C.H. Hu, A.J. Dare, A.M. James, S. Rogatti, R.C. Hartley, S. Eaton, A.S. Costa, P.S. Brookes, S.M. Davidson, M.R. Duchen, K. Saeb-Parsy, M.J. Shattock, A.J. Robinson, L.M. Work, C. Frezza, T. Krieg, M.P. Murphy, Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS, Nature 515(7527) (2014) 431-5.
[49] E.L. Robb, J.M. Gawel, D. Aksentijevic, H.M. Cocheme, T.S. Stewart, M.M. Shchepinova, H. Qiang, T.A. Prime, T.P. Bright, A.M. James, M.J. Shattock, H.M. Senn, R.C. Hartley, M.P. Murphy, Selective superoxide generation within mitochondria by the targeted redox cycler MitoParaquat, Free Radic Biol Med 89 (2015) 883-94.
[50] A. Sanz, P. Caro, G. Barja, Protein restriction without strong caloric restriction decreases mitochondrial oxygen radical production and oxidative DNA damage in rat liver, J Bioenerg Biomembr 36(6) (2004) 545-52.
[51] V. Ayala, A. Naudi, A. Sanz, P. Caro, M. Portero-Otin, G. Barja, R. Pamplona, Dietary protein restriction decreases oxidative protein damage, peroxidizability index, and mitochondrial complex I content in rat liver, J Gerontol A Biol Sci Med Sci 62(4) (2007) 352-60.
[52] A.J. Lambert, J.A. Buckingham, H.M. Boysen, M.D. Brand, Low complex I content explains the low hydrogen peroxide production rate of heart mitochondria from the long-lived pigeon, Columba livia, Aging Cell 9(1) (2010) 78-91.
[53] R. Stefanatos, A. Sanz, Mitochondrial complex I: a central regulator of the aging process, Cell Cycle 10(10) (2011) 1528-32.
[54] C.L. Quinlan, A.L. Orr, I.V. Perevoshchikova, J.R. Treberg, B.A. Ackrell, M.D. Brand, Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions, J Biol Chem 287(32) (2012) 27255-64.
[55] H.S. Wong, P.A. Dighe, V. Mezera, P.A. Monternier, M.D. Brand, Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions, J Biol Chem 292(41) (2017) 16804-16809.
[56] R.L. Goncalves, C.L. Quinlan, I.V. Perevoshchikova, M. Hey-Mogensen, M.D. Brand, Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise, J Biol Chem 290(1) (2015) 209-27.
[57] C.L. Quinlan, J.R. Treberg, I.V. Perevoshchikova, A.L. Orr, M.D. Brand, Native rates of superoxide production from multiple sites in isolated mitochondria measured using endogenous reporters, Free Radic Biol Med 53(9) (2012) 1807-17.
[58] S. Miwa, A. Treumann, A. Bell, G. Vistoli, G. Nelson, S. Hay, T. von Zglinicki, Carboxylesterase converts Amplex red to resorufin: Implications for mitochondrial H2O2 release assays, Free Radic Biol Med 90 (2016) 173-83.

[59] K. Hadrava Vanova, M. Kraus, J. Neuzil, J. Rohlena, Mitochondrial complex II and reactive oxygen species in disease and therapy, Redox report : communications in free radical research 25(1) (2020) 26-32.
[60] T.P. Keeley, G.E. Mann, Defining Physiological Normoxia for Improved Translation of Cell Physiology to Animal Models and Humans, Physiol Rev 99(1) (2019) 161-234.
[61] R.L.S. Goncalves, M.A. Watson, H.S. Wong, A.L. Orr, M.D. Brand, The use of site-specific suppressors to measure the relative contributions of different mitochondrial sites to skeletal muscle superoxide and hydrogen peroxide production, Redox biology 28 (2020) 101341.
[62] J. Fang, H.S. Wong, M.D. Brand, Production of superoxide and hydrogen peroxide in the mitochondrial matrix is dominated by site IQ of complex I in diverse cell lines, Redox biology 37 (2020) 101722.
[63] G.C. Brown, V. Borutaite, There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells, Mitochondrion 12(1) (2012) 1-4.
[64] M.D. Brand, R.L. Goncalves, A.L. Orr, L. Vargas, A.A. Gerencser, M. Borch Jensen, Y.T. Wang, S. Melov, C.N. Turk, J.T. Matzen, V.J. Dardov, H.M. Petrassi, S.L. Meeusen, I.V. Perevoshchikova, H. Jasper, P.S. Brookes, E.K. Ainscow, Suppressors of Superoxide-H2O2 Production at Site IQ of Mitochondrial Complex I Protect against Stem Cell Hyperplasia and Ischemia-Reperfusion Injury, Cell Metab 24(4) (2016) 582-592.
[65] M.D. Brand, The sites and topology of mitochondrial superoxide production, Exp Gerontol 45(7-8) (2010) 466-72.
[66] E.L. Robb, A.R. Hall, T.A. Prime, S. Eaton, M. Szibor, C. Viscomi, A.M. James, M.P. Murphy, Control of mitochondrial superoxide production by reverse electron transport at complex I, J Biol Chem 293(25) (2018) 9869-9879.
[67] I. Arias-Mayenco, P. Gonzalez-Rodriguez, H. Torres-Torrelo, L. Gao, M.C. Fernandez-Aguera, V. Bonilla-Henao, P. Ortega-Saenz, J. Lopez-Barneo, Acute O2 Sensing: Role of Coenzyme QH2/Q Ratio and Mitochondrial ROS Compartmentalization, Cell Metab 28(1) (2018) 145-158 e4.
[68] B. Chance, G. Hollunger, The interaction of energy and electron transfer reactions in mitochondria. I. General properties and nature of the products of succinate-linked reduction of pyridine nucleotide, J Biol Chem 236 (1961) 1534-43.
[69] J. Hirst, M.S. King, K.R. Pryde, The production of reactive oxygen species by complex I, Biochem Soc Trans 36(Pt 5) (2008) 976-80.
[70] J. Hirst, M.M. Roessler, Energy conversion, redox catalysis and generation of reactive oxygen species by respiratory complex I, Biochim Biophys Acta 1857(7) (2016) 872-83.
[71] E.T. Chouchani, V.R. Pell, A.M. James, L.M. Work, K. Saeb-Parsy, C. Frezza, T. Krieg, M.P. Murphy, A Unifying Mechanism for Mitochondrial Superoxide Production during Ischemia-Reperfusion Injury, Cell Metab (2016).
[72] S. Miwa, J. St-Pierre, L. Partridge, M.D. Brand, Superoxide and hydrogen peroxide production by Drosophila mitochondria, Free Radic Biol Med 35(8) (2003) 938-48.
[73] R.J. Mailloux, A. Young, J. Chalker, D. Gardiner, M. O'Brien, L. Slade, J.T. Brosnan, Choline and dimethylglycine produce superoxide/hydrogen peroxide from the electron transport chain in liver mitochondria, FEBS Lett 590(23) (2016) 4318-4328.
[74] M.C. Fernandez-Aguera, L. Gao, P. Gonzalez-Rodriguez, C.O. Pintado, I. Arias-Mayenco, P. Garcia-Flores, A. Garcia-Perganeda, A. Pascual, P. Ortega-Saenz, J. Lopez-Barneo, Oxygen Sensing by Arterial Chemoreceptors Depends on Mitochondrial Complex I Signaling, Cell Metab 22(5) (2015) 825-37.

[75] E.L. Mills, B. Kelly, A. Logan, A.S. Costa, M. Varma, C.E. Bryant, P. Tourlomousis, J.H. Dabritz, E. Gottlieb, I. Latorre, S.C. Corr, G. McManus, D. Ryan, H.T. Jacobs, M. Szibor, R.J. Xavier, T. Braun, C. Frezza, M.P. Murphy, L.A. O'Neill, Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages, Cell 167(2) (2016) 457-470 e13.
[76] S. Bahadorani, J. Cho, T. Lo, H. Contreras, H.O. Lawal, D.E. Krantz, T.J. Bradley, D.W. Walker, Neuronal expression of a single-subunit yeast NADH-ubiquinone oxidoreductase (Ndi1) extends Drosophila lifespan, Aging Cell 9(2) (2010) 191-202.
[77] F. Scialo, A. Sriram, A. Naudi, V. Ayala, M. Jove, R. Pamplona, A. Sanz, Target of rapamycin activation predicts lifespan in fruit flies, Cell Cycle (2015) 0.
[78] L. Bleier, S. Drose, Superoxide generation by complex III: from mechanistic rationales to functional consequences, Biochim Biophys Acta 1827(11-12) (2013) 1320-31.
[79] A. Sanz, R. Stefanatos, G. McIlroy, Production of reactive oxygen species by the mitochondrial electron transport chain in Drosophila melanogaster, J Bioenerg Biomembr 42(2) (2010) 135-42.
[80] R.D. Guzy, B. Hoyos, E. Robin, H. Chen, L. Liu, K.D. Mansfield, M.C. Simon, U. Hammerling, P.T. Schumacker, Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing, Cell Metab 1(6) (2005) 401-8.
[81] P. Hernansanz-Agustin, C. Choya-Foces, S. Carregal-Romero, E. Ramos, T. Oliva, T. Villa-Pina, L. Moreno, A. Izquierdo-Alvarez, J.D. Cabrera-Garcia, A. Cortes, A.V. Lechuga-Vieco, P. Jadiya, E. Navarro, E. Parada, A. Palomino-Antolin, D. Tello, R. Acin-Perez, J.C. Rodriguez-Aguilera, P. Navas, A. Cogolludo, I. Lopez-Montero, A. Martinez-Del-Pozo, J. Egea, M.G. Lopez, J.W. Elrod, J. Ruiz-Cabello, A. Bogdanova, J.A. Enriquez, A. Martinez-Ruiz, Na(+) controls hypoxic signalling by the mitochondrial respiratory chain, Nature 586(7828) (2020) 287-291.
[82] F.L. Muller, Y. Liu, H. Van Remmen, Complex III releases superoxide to both sides of the inner mitochondrial membrane, J Biol Chem 279(47) (2004) 49064-73.
[83] A.E. McDonald, D.V. Gospodaryov, Alternative NAD(P)H dehydrogenase and alternative oxidase: Proposed physiological roles in animals, Mitochondrion 45 (2019) 7-17.
[84] S. Saari, G.S. Garcia, K. Bremer, M.M. Chioda, A. Andjelkovic, P.V. Debes, M. Nikinmaa, M. Szibor, E. Dufour, P. Rustin, M.T. Oliveira, H.T. Jacobs, Alternative respiratory chain enzymes: Therapeutic potential and possible pitfalls, Biochim Biophys Acta Mol Basis Dis 1865(4) (2019) 854-866.
[85] A. Sanz, D.J. Fernandez-Ayala, R.K. Stefanatos, H.T. Jacobs, Mitochondrial ROS production correlates with, but does not directly regulate lifespan in Drosophila, Aging (Albany NY) 2(4) (2010) 200-23.
[86] G.A. Hakkaart, E.P. Dassa, H.T. Jacobs, P. Rustin, Allotopic expression of a mitochondrial alternative oxidase confers cyanide resistance to human cell respiration, EMBO Rep 7(3) (2006) 341-5.
[87] M. Szibor, T. Gainutdinov, E. Fernandez-Vizarra, E. Dufour, Z. Gizatullina, G. Debska-Vielhaber, J. Heidler, I. Wittig, C. Viscomi, F. Gellerich, A.L. Moore, Bioenergetic consequences from xenotopic expression of a tunicate AOX in mouse mitochondria: Switch from RET and ROS to FET, Biochim Biophys Acta Bioenerg 1861(2) (2020) 148137.
[88] B. May, L. Young, A.L. Moore, Structural insights into the alternative oxidases: are all oxidases made equal?, Biochem Soc Trans 45(3) (2017) 731-740.
[89] E. Perales-Clemente, M.P. Bayona-Bafaluy, A. Perez-Martos, A. Barrientos, P. Fernandez-Silva, J.A. Enriquez, Restoration of electron transport without proton pumping in mammalian mitochondria, Proc Natl Acad Sci U S A 105(48) (2008) 18735-9.

[90] D.J. Fernandez-Ayala, A. Sanz, S. Vartiainen, K.K. Kemppainen, M. Babusiak, E. Mustalahti, R. Costa, T. Tuomela, M. Zeviani, J. Chung, K.M. O'Dell, P. Rustin, H.T. Jacobs, Expression of the Ciona intestinalis alternative oxidase (AOX) in Drosophila complements defects in mitochondrial oxidative phosphorylation, Cell Metab 9(5) (2009) 449-60.
[91] R. El-Khoury, E. Dufour, M. Rak, N. Ramanantsoa, N. Grandchamp, Z. Csaba, B. Duvillie, P. Benit, J. Gallego, P. Gressens, C. Sarkis, H.T. Jacobs, P. Rustin, Alternative oxidase expression in the mouse enables bypassing cytochrome c oxidase blockade and limits mitochondrial ROS overproduction, PLoS Genet 9(1) (2013) e1003182.
[92] K.K. Kemppainen, J. Rinne, A. Sriram, M. Lakanmaa, A. Zeb, T. Tuomela, A. Popplestone, S. Singh, A. Sanz, P. Rustin, H.T. Jacobs, Expression of alternative oxidase in Drosophila ameliorates diverse phenotypes due to cytochrome oxidase deficiency, Hum Mol Genet 23(8) (2014) 2078-93.
[93] J. Rajendran, J. Purhonen, S. Tegelberg, O.P. Smolander, M. Morgelin, J. Rozman, V. Gailus-Durner, H. Fuchs, M. Hrabe de Angelis, P. Auvinen, E. Mervaala, H.T. Jacobs, M. Szibor, V. Fellman, J. Kallijarvi, Alternative oxidase-mediated respiration prevents lethal mitochondrial cardiomyopathy, EMBO Mol Med 11(1) (2019).
[94] A. Andjelkovic, A. Mordas, L. Bruinsma, A. Ketola, G. Cannino, L. Giordano, P.K. Dhandapani, M. Szibor, E. Dufour, H.T. Jacobs, Expression of the Alternative Oxidase Influences Jun N-Terminal Kinase Signaling and Cell Migration, Mol Cell Biol 38(24) (2018).
[95] K.K. Kemppainen, E. Kemppainen, H.T. Jacobs, The alternative oxidase AOX does not rescue the phenotype of tko25t mutant flies, G3 4(10) (2014) 2013-21.
[96] S. Vartiainen, S. Chen, J. George, T. Tuomela, K.R. Luoto, K.M. O'Dell, H.T. Jacobs, Phenotypic rescue of a Drosophila model of mitochondrial ANT1 disease, Dis Model Mech 7(6) (2014) 635-48.
[97] A.P.C. Rodrigues, A.F. Camargo, A. Andjelkovic, H.T. Jacobs, M.T. Oliveira, Developmental arrest in Drosophila melanogaster caused by mitochondrial DNA replication defects cannot be rescued by the alternative oxidase, Scientific reports 8(1) (2018) 10882.
[98] S.A. Dogan, R. Cerutti, C. Beninca, G. Brea-Calvo, H.T. Jacobs, M. Zeviani, M. Szibor, C. Viscomi, Perturbed Redox Signaling Exacerbates a Mitochondrial Myopathy, Cell Metab 28(5) (2018) 764-775 e5.
[99] M. Szibor, R. Schreckenberg, Z. Gizatullina, E. Dufour, M. Wiesnet, P.K. Dhandapani, G. Debska-Vielhaber, J. Heidler, I. Wittig, T.A. Nyman, U. Gartner, A.R. Hall, V. Pell, C. Viscomi, T. Krieg, M.P. Murphy, T. Braun, F.N. Gellerich, K.D. Schluter, H.T. Jacobs, Respiratory chain signalling is essential for adaptive remodelling following cardiac ischaemia, J Cell Mol Med 24(6) (2020) 3534-3548.
[100] A. Sanz, M. Soikkeli, M. Portero-Otin, A. Wilson, E. Kemppainen, G. McIlroy, S. Ellila, K.K. Kemppainen, T. Tuomela, M. Lakanmaa, E. Kiviranta, R. Stefanatos, E. Dufour, B. Hutz, A. Naudi, M. Jove, A. Zeb, S. Vartiainen, A. Matsuno-Yagi, T. Yagi, P. Rustin, R. Pamplona, H.T. Jacobs, Expression of the yeast NADH dehydrogenase Ndi1 in Drosophila confers increased lifespan independently of dietary restriction, Proc Natl Acad Sci U S A 107(20) (2010) 9105-10.
[101] D.V. Gospodaryov, O.V. Lushchak, B.M. Rovenko, N.V. Perkhulyn, M. Gerards, T. Tuomela, H.T. Jacobs, Ciona intestinalis NADH dehydrogenase NDX confers stress-resistance and extended lifespan on Drosophila, Biochim Biophys Acta 1837(11) (2014) 1861-1869.
[102] D.V. Gospodaryov, O.M. Strilbytska, U.V. Semaniuk, N.V. Perkhulyn, B.M. Rovenko, I.S. Yurkevych, A.G. Barata, T.P. Dick, O.V. Lushchak, H.T. Jacobs, Alternative NADH

dehydrogenase extends lifespan and increases resistance to xenobiotics in Drosophila, Biogerontology 21(2) (2020) 155-171.
[103] W.W. Wheaton, S.E. Weinberg, R.B. Hamanaka, S. Soberanes, L.B. Sullivan, E. Anso, A. Glasauer, E. Dufour, G.M. Mutlu, G.S. Budigner, N.S. Chandel, Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis, eLife 3 (2014) e02242.
[104] B.B. Seo, J. Wang, T.R. Flotte, T. Yagi, A. Matsuno-Yagi, Use of the NADH-quinone oxidoreductase (NDI1) gene of Saccharomyces cerevisiae as a possible cure for complex I defects in human cells, J Biol Chem 275(48) (2000) 37774-8.
[105] J. Cho, J.H. Hur, J. Graniel, S. Benzer, D.W. Walker, Expression of yeast NDI1 rescues a Drosophila complex I assembly defect, PLoS One 7(11) (2012) e50644.
[106] G.S. McElroy, C.R. Reczek, P.A. Reyfman, D.S. Mithal, C.M. Horbinski, N.S. Chandel, NAD+ Regeneration Rescues Lifespan, but Not Ataxia, in a Mouse Model of Brain Mitochondrial Complex I Dysfunction, Cell Metab 32(2) (2020) 301-308 e6.
[107] S. Vilain, G. Esposito, D. Haddad, O. Schaap, M.P. Dobreva, M. Vos, S. Van Meensel, V.A. Morais, B. De Strooper, P. Verstreken, The yeast complex I equivalent NADH dehydrogenase rescues pink1 mutants, PLoS Genet 8(1) (2012) e1002456.
[108] M. Marella, B.B. Seo, T. Yagi, A. Matsuno-Yagi, Parkinson's disease and mitochondrial complex I: a perspective on the Ndi1 therapy, J Bioenerg Biomembr 41(6) (2009) 493-7.
[109] J. Barber-Singh, B.B. Seo, E. Nakamaru-Ogiso, Y.S. Lau, A. Matsuno-Yagi, T. Yagi, Neuroprotective effect of long-term NDI1 gene expression in a chronic mouse model of Parkinson disorder, Rejuvenation Res 12(4) (2009) 259-67.
[110] R.M. Mentzer, Jr., J. Wider, C.N. Perry, R.A. Gottlieb, Reduction of infarct size by the therapeutic protein TAT-Ndi1 in vivo, J Cardiovasc Pharmacol Ther 19(3) (2014) 315-20.
[111] S. Lee, E. Tak, J. Lee, M.A. Rashid, M.P. Murphy, J. Ha, S.S. Kim, Mitochondrial H2O2 generated from electron transport chain complex I stimulates muscle differentiation, Cell Res 21(5) (2011) 817-34.
[112] T.E. Beach, H.A. Prag, L. Pala, A. Logan, M.M. Huang, A.V. Gruszczyk, J.L. Martin, K. Mahbubani, M.O. Hamed, S.A. Hosgood, M.L. Nicholson, A.M. James, R.C. Hartley, M.P. Murphy, K. Saeb-Parsy, Targeting succinate dehydrogenase with malonate ester prodrugs decreases renal ischemia reperfusion injury, Redox biology 36 (2020) 101640.
[113] J.L. Martin, A.S.H. Costa, A.V. Gruszczyk, T.E. Beach, F.M. Allen, H.A. Prag, E.C. Hinchy, K. Mahbubani, M. Hamed, L. Tronci, E. Nikitopoulou, A.M. James, T. Krieg, A.J. Robinson, M.M. Huang, S.T. Caldwell, A. Logan, L. Pala, R.C. Hartley, C. Frezza, K. Saeb-Parsy, M.P. Murphy, Succinate accumulation drives ischaemia-reperfusion injury during organ transplantation, Nat Metab 1 (2019) 966-974.
[114] R. Moreno-Loshuertos, J.A. Enriquez, Respiratory supercomplexes and the functional segmentation of the CoQ pool, Free Radic Biol Med 100 (2016) 5-13.
[115] J.G. Fedor, J. Hirst, Mitochondrial Supercomplexes Do Not Enhance Catalysis by Quinone Channeling, Cell Metab 28(3) (2018) 525-531 e4.
[116] E. Calvo, S. Cogliati, P. Hernansanz-Agustin, M. Loureiro-Lopez, A. Guaras, R.A. Casuso, F. Garcia-Marques, R. Acin-Perez, Y. Marti-Mateos, J.C. Silla-Castro, M. Carro-Alvarellos, J.R. Huertas, J. Vazquez, J.A. Enriquez, Functional role of respiratory supercomplexes in mice: SCAF1 relevance and segmentation of the Qpool, Sci Adv 6(26) (2020) eaba7509.
[117] E. Lapuente-Brun, R. Moreno-Loshuertos, R. Acin-Perez, A. Latorre-Pellicer, C. Colas, E. Balsa, E. Perales-Clemente, P.M. Quiros, E. Calvo, M.A. Rodriguez-Hernandez, P. Navas, R. Cruz, A. Carracedo, C. Lopez-Otin, A. Perez-Martos, P. Fernandez-Silva, E.

Fernandez-Vizarra, J.A. Enriquez, Supercomplex assembly determines electron flux in the mitochondrial electron transport chain, Science 340(6140) (2013) 1567-70.
[118] Q. Chen, E.J. Vazquez, S. Moghaddas, C.L. Hoppel, E.J. Lesnefsky, Production of reactive oxygen species by mitochondria: central role of complex III, J Biol Chem 278(38) (2003) 36027-31.
[119] R. Gredilla, A. Sanz, M. Lopez-Torres, G. Barja, Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart, FASEB J 15(9) (2001) 1589-91.
[120] R. Stefanatos, A. Sriram, E. Kiviranta, A. Mohan, V. Ayala, H.T. Jacobs, R. Pamplona, A. Sanz, dj-1beta regulates oxidative stress, insulin-like signaling and development in Drosophila melanogaster, Cell Cycle 11(20) (2012) 3876-86.
[121] G.S. McElroy, N.S. Chandel, Mitochondria control acute and chronic responses to hypoxia, Exp Cell Res 356(2) (2017) 217-222.
[122] N.S. Chandel, D.S. McClintock, C.E. Feliciano, T.M. Wood, J.A. Melendez, A.M. Rodriguez, P.T. Schumacker, Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing, J Biol Chem 275(33) (2000) 25130-8.
[123] A.L. Orr, L. Vargas, C.N. Turk, J.E. Baaten, J.T. Matzen, V.J. Dardov, S.J. Attle, J. Li, D.C. Quackenbush, R.L. Goncalves, I.V. Perevoshchikova, H.M. Petrassi, S.L. Meeusen, E.K. Ainscow, M.D. Brand, Suppressors of superoxide production from mitochondrial complex III, Nat Chem Biol 11(11) (2015) 834-6.
[124] G.B. Waypa, J.D. Marks, R.D. Guzy, P.T. Mungai, J.M. Schriewer, D. Dokic, M.K. Ball, P.T. Schumacker, Superoxide generated at mitochondrial complex III triggers acute responses to hypoxia in the pulmonary circulation, Am J Respir Crit Care Med 187(4) (2013) 424-32.
[125] N. Sommer, N. Alebrahimdehkordi, O. Pak, F. Knoepp, I. Strielkov, S. Scheibe, E. Dufour, A. Andjelkovic, A. Sydykov, A. Saraji, A. Petrovic, K. Quanz, M. Hecker, M. Kumar, J. Wahl, S. Kraut, W. Seeger, R.T. Schermuly, H.A. Ghofrani, K. Ramser, T. Braun, H.T. Jacobs, N. Weissmann, M. Szibor, Bypassing mitochondrial complex III using alternative oxidase inhibits acute pulmonary oxygen sensing, Sci Adv 6(16) (2020) eaba0694.
[126] L.A. Sena, S. Li, A. Jairaman, M. Prakriya, T. Ezponda, D.A. Hildeman, C.R. Wang, P.T. Schumacker, J.D. Licht, H. Perlman, P.J. Bryce, N.S. Chandel, Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling, Immunity 38(2) (2013) 225-36.
[127] E. Seki, S. De Minicis, C.H. Osterreicher, J. Kluwe, Y. Osawa, D.A. Brenner, R.F. Schwabe, TLR4 enhances TGF-beta signaling and hepatic fibrosis, Nature medicine 13(11) (2007) 1324-32.
[128] M. Jain, S. Rivera, E.A. Monclus, L. Synenki, A. Zirk, J. Eisenbart, C. Feghali-Bostwick, G.M. Mutlu, G.R. Budinger, N.S. Chandel, Mitochondrial reactive oxygen species regulate transforming growth factor-beta signaling, J Biol Chem 288(2) (2013) 770-7.
[129] K.V. Tormos, E. Anso, R.B. Hamanaka, J. Eisenbart, J. Joseph, B. Kalyanaraman, N.S. Chandel, Mitochondrial complex III ROS regulate adipocyte differentiation, Cell Metab 14(4) (2011) 537-44.
[130] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, Nature reviews. Drug discovery 8(7) (2009) 579-91.
[131] I. Martinez-Reyes, L.R. Cardona, H. Kong, K. Vasan, G.S. McElroy, M. Werner, H. Kihshen, C.R. Reczek, S.E. Weinberg, P. Gao, E.M. Steinert, R. Piseaux, G.R.S. Budinger,

N.S. Chandel, Mitochondrial ubiquinol oxidation is necessary for tumour growth, Nature 585(7824) (2020) 288-292.
[132] S. Soberanes, D. Urich, C.M. Baker, Z. Burgess, S.E. Chiarella, E.L. Bell, A.J. Ghio, A. De Vizcaya-Ruiz, J. Liu, K.M. Ridge, D.W. Kamp, N.S. Chandel, P.T. Schumacker, G.M. Mutlu, G.R. Budinger, Mitochondrial complex III-generated oxidants activate ASK1 and JNK to induce alveolar epithelial cell death following exposure to particulate matter air pollution, J Biol Chem 284(4) (2009) 2176-86.
[133] S. Soberanes, A.V. Misharin, A. Jairaman, L. Morales-Nebreda, A.C. McQuattie-Pimentel, T. Cho, R.B. Hamanaka, A.Y. Meliton, P.A. Reyfman, J.M. Walter, C.I. Chen, M. Chi, S. Chiu, F.J. Gonzalez-Gonzalez, M. Antalek, H. Abdala-Valencia, S.E. Chiarella, K.A. Sun, P.S. Woods, A.J. Ghio, M. Jain, H. Perlman, K.M. Ridge, R.I. Morimoto, J.I. Sznajder, W.E. Balch, S.M. Bhorade, A. Bharat, M. Prakriya, N.S. Chandel, G.M. Mutlu, G.R.S. Budinger, Metformin Targets Mitochondrial Electron Transport to Reduce Air-Pollution-Induced Thrombosis, Cell Metab 29(2) (2019) 335-347 e5.
[134] L. Giordano, A. Farnham, P.K. Dhandapani, L. Salminen, J. Bhaskaran, R. Voswinckel, P. Rauschkolb, S. Scheibe, N. Sommer, C. Beisswenger, N. Weissmann, T. Braun, H.T. Jacobs, R. Bals, C. Herr, M. Szibor, Alternative Oxidase Attenuates Cigarette Smoke-induced Lung Dysfunction and Tissue Damage, Am J Respir Cell Mol Biol 60(5) (2019) 515-522.
[135] T.L. Dawson, G.J. Gores, A.L. Nieminen, B. Herman, J.J. Lemasters, Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes, Am J Physiol 264(4 Pt 1) (1993) C961-7.
[136] A. Navarro, A. Boveris, Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging, Am J Physiol Regul Integr Comp Physiol 287(5) (2004) R1244-9.
[137] P. Nicholls, D.C. Marshall, C.E. Cooper, M.T. Wilson, Sulfide inhibition of and metabolism by cytochrome c oxidase, Biochem Soc Trans 41(5) (2013) 1312-6.
[138] Y. Wei, C. Kenyon, Roles for ROS and hydrogen sulfide in the longevity response to germline loss in Caenorhabditis elegans, Proc Natl Acad Sci U S A 113(20) (2016) E2832-41.
[139] C. Hine, E. Harputlugil, Y. Zhang, C. Ruckenstuhl, B.C. Lee, L. Brace, A. Longchamp, J.H. Trevino-Villarreal, P. Mejia, C.K. Ozaki, R. Wang, V.N. Gladyshev, F. Madeo, W.B. Mair, J.R. Mitchell, Endogenous hydrogen sulfide production is essential for dietary restriction benefits, Cell 160(1-2) (2015) 132-44.
[140] S. Moncada, J.D. Erusalimsky, Does nitric oxide modulate mitochondrial energy generation and apoptosis?, Nat Rev Mol Cell Biol 3(3) (2002) 214-20.
[141] M. Palacios-Callender, M. Quintero, V.S. Hollis, R.J. Springett, S. Moncada, Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome c oxidase, Proc Natl Acad Sci U S A 101(20) (2004) 7630-5.
[142] B.S. Zuckerbraun, B.Y. Chin, M. Bilban, J.C. d'Avila, J. Rao, T.R. Billiar, L.E. Otterbein, Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species, FASEB J 21(4) (2007) 1099-106.
[143] K.J. Pearson, K.N. Lewis, N.L. Price, J.W. Chang, E. Perez, M.V. Cascajo, K.L. Tamashiro, S. Poosala, A. Csiszar, Z. Ungvari, T.W. Kensler, M. Yamamoto, J.M. Egan, D.L. Longo, D.K. Ingram, P. Navas, R. de Cabo, Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction, Proc Natl Acad Sci U S A 105(7) (2008) 2325-30.
[144] H.B. Suliman, M.S. Carraway, L.G. Tatro, C.A. Piantadosi, A new activating role for CO in cardiac mitochondrial biogenesis, Journal of cell science 120(Pt 2) (2007) 299-308.

[145] W.W. Chen, K. Birsoy, M.M. Mihaylova, H. Snitkin, I. Stasinski, B. Yucel, E.C. Bayraktar, J.E. Carette, C.B. Clish, T.R. Brummelkamp, D.D. Sabatini, D.M. Sabatini, Inhibition of ATPIF1 ameliorates severe mitochondrial respiratory chain dysfunction in mammalian cells, Cell reports 7(1) (2014) 27-34.
[146] M. Campanella, E. Casswell, S. Chong, Z. Farah, M.R. Wieckowski, A.Y. Abramov, A. Tinker, M.R. Duchen, Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1, Cell Metab 8(1) (2008) 13-25.
[147] L. Sanchez-Cenizo, L. Formentini, M. Aldea, A.D. Ortega, P. Garcia-Huerta, M. Sanchez-Arago, J.M. Cuezva, Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype, J Biol Chem 285(33) (2010) 25308-13.
[148] L. Formentini, M. Sanchez-Arago, L. Sanchez-Cenizo, J.M. Cuezva, The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response, Mol Cell 45(6) (2012) 731-42.
[149] G. Santamaria, M. Martinez-Diez, I. Fabregat, J.M. Cuezva, Efficient execution of cell death in non-glycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H+-ATP synthase, Carcinogenesis 27(5) (2006) 925-35.
[150] L. Formentini, M.P. Pereira, L. Sanchez-Cenizo, F. Santacatterina, J.J. Lucas, C. Navarro, A. Martinez-Serrano, J.M. Cuezva, In vivo inhibition of the mitochondrial H+-ATP synthase in neurons promotes metabolic preconditioning, EMBO J 33(7) (2014) 762-78.
[151] L. Formentini, F. Santacatterina, C. Nunez de Arenas, K. Stamatakis, D. Lopez-Martinez, A. Logan, M. Fresno, R. Smits, M.P. Murphy, J.M. Cuezva, Mitochondrial ROS Production Protects the Intestine from Inflammation through Functional M2 Macrophage Polarization, Cell reports 19(6) (2017) 1202-1213.
[152] X. Sun, C.T. Wheeler, J. Yolitz, M. Laslo, T. Alberico, Y. Sun, Q. Song, S. Zou, A mitochondrial ATP synthase subunit interacts with TOR signaling to modulate protein homeostasis and lifespan in Drosophila, Cell reports 8(6) (2014) 1781-92.
[153] S. Bahadorani, J.H. Hur, T. Lo, Jr., K. Vu, D.W. Walker, Perturbation of mitochondrial complex V alters the response to dietary restriction in Drosophila, Aging Cell 9(1) (2010) 100-3.
[154] J. Goldberg, A. Currais, M. Prior, W. Fischer, C. Chiruta, E. Ratliff, D. Daugherty, R. Dargusch, K. Finley, P.B. Esparza-Molto, J.M. Cuezva, P. Maher, M. Petrascheck, D. Schubert, The mitochondrial ATP synthase is a shared drug target for aging and dementia, Aging Cell 17(2) (2018).
[155] R.M. Chin, X. Fu, M.Y. Pai, L. Vergnes, H. Hwang, G. Deng, S. Diep, B. Lomenick, V.S. Meli, G.C. Monsalve, E. Hu, S.A. Whelan, J.X. Wang, G. Jung, G.M. Solis, F. Fazlollahi, C. Kaweeteerawat, A. Quach, M. Nili, A.S. Krall, H.A. Godwin, H.R. Chang, K.F. Faull, F. Guo, M. Jiang, S.A. Trauger, A. Saghatelian, D. Braas, H.R. Christofk, C.F. Clarke, M.A. Teitell, M. Petrascheck, K. Reue, M.E. Jung, A.R. Frand, J. Huang, The metabolite alpha-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR, Nature 510(7505) (2014) 397-401.
[156] A. Asadi Shahmirzadi, D. Edgar, C.Y. Liao, Y.M. Hsu, M. Lucanic, A. Asadi Shahmirzadi, C.D. Wiley, G. Gan, D.E. Kim, H.G. Kasler, C. Kuehnemann, B. Kaplowitz, D. Bhaumik, R.R. Riley, B.K. Kennedy, G.J. Lithgow, Alpha-Ketoglutarate, an Endogenous Metabolite, Extends Lifespan and Compresses Morbidity in Aging Mice, Cell Metab 32(3) (2020) 447-456 e6.
[157] E. Naik, V.M. Dixit, Mitochondrial reactive oxygen species drive proinflammatory cytokine production, J Exp Med 208(3) (2011) 417-20.

[158] D. Bazopoulou, D. Knoefler, Y. Zheng, K. Ulrich, B.J. Oleson, L. Xie, M. Kim, A. Kaufmann, Y.T. Lee, Y. Dou, Y. Chen, S. Quan, U. Jakob, Developmental ROS individualizes organismal stress resistance and lifespan, Nature 576(7786) (2019) 301-305.
[159] H.M. Cocheme, C. Quin, S.J. McQuaker, F. Cabreiro, A. Logan, T.A. Prime, I. Abakumova, J.V. Patel, I.M. Fearnley, A.M. James, C.M. Porteous, R.A. Smith, S. Saeed, J.E. Carre, M. Singer, D. Gems, R.C. Hartley, L. Partridge, M.P. Murphy, Measurement of H2O2 within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix, Cell Metab 13(3) (2011) 340-50.
[160] S. Miwa, H. Jow, K. Baty, A. Johnson, R. Czapiewski, G. Saretzki, A. Treumann, T. von Zglinicki, Low abundance of the matrix arm of complex I in mitochondria predicts longevity in mice, Nature communications 5 (2014) 3837.
[161] C. Correia-Melo, F.D. Marques, R. Anderson, G. Hewitt, R. Hewitt, J. Cole, B.M. Carroll, S. Miwa, J. Birch, A. Merz, M.D. Rushton, M. Charles, D. Jurk, S.W. Tait, R. Czapiewski, L. Greaves, G. Nelson, Y.M. Bohlooly, S. Rodriguez-Cuenca, A. Vidal-Puig, D. Mann, G. Saretzki, G. Quarato, D.R. Green, P.D. Adams, T. von Zglinicki, V.I. Korolchuk, J.F. Passos, Mitochondria are required for pro-ageing features of the senescent phenotype, EMBO J 35(7) (2016) 724-42.
[162] M. Lopez-Torres, R. Gredilla, A. Sanz, G. Barja, Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria, Free Radic Biol Med 32(9) (2002) 882-9.
[163] B. Drew, S. Phaneuf, A. Dirks, C. Selman, R. Gredilla, A. Lezza, G. Barja, C. Leeuwenburgh, Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart, Am J Physiol Regul Integr Comp Physiol 284(2) (2003) R474-80.
[164] A. Sanz, P. Caro, J. Ibanez, J. Gomez, R. Gredilla, G. Barja, Dietary restriction at old age lowers mitochondrial oxygen radical production and leak at complex I and oxidative DNA damage in rat brain, J Bioenerg Biomembr 37(2) (2005) 83-90.
[165] J.F. Passos, G. Saretzki, S. Ahmed, G. Nelson, T. Richter, H. Peters, I. Wappler, M.J. Birket, G. Harold, K. Schaeuble, M.A. Birch-Machin, T.B. Kirkwood, T. von Zglinicki, Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence, PLoS Biol 5(5) (2007) e110.
[166] V.I. Korolchuk, S. Miwa, B. Carroll, T. von Zglinicki, Mitochondria in Cell Senescence: Is Mitophagy the Weakest Link?, EBioMedicine 21 (2017) 7-13.
[167] J. Jacobson, A.J. Lambert, M. Portero-Otin, R. Pamplona, T. Magwere, S. Miwa, Y. Driege, M.D. Brand, L. Partridge, Biomarkers of aging in Drosophila, Aging Cell 9(4) (2010) 466-77.
[168] P. Jenner, Oxidative stress in Parkinson's disease, Ann Neurol 53 Suppl 3 (2003) S26-36; discussion S36-8.
[169] K. Hirai, G. Aliev, A. Nunomura, H. Fujioka, R.L. Russell, C.S. Atwood, A.B. Johnson, Y. Kress, H.V. Vinters, M. Tabaton, S. Shimohama, A.D. Cash, S.L. Siedlak, P.L. Harris, P.K. Jones, R.B. Petersen, G. Perry, M.A. Smith, Mitochondrial abnormalities in Alzheimer's disease, J Neurosci 21(9) (2001) 3017-23.
[170] M. Panel, B. Ghaleh, D. Morin, Mitochondria and aging: A role for the mitochondrial transition pore?, Aging Cell 17(4) (2018) e12793.
[171] S. Moghaddas, C.L. Hoppel, E.J. Lesnefsky, Aging defect at the QO site of complex III augments oxyradical production in rat heart interfibrillar mitochondria, Arch Biochem Biophys 414(1) (2003) 59-66.

[172] E. Rodriguez-Bies, P. Navas, G. Lopez-Lluch, Age-dependent effect of every-other-day feeding and aerobic exercise in ubiquinone levels and related antioxidant activities in mice muscle, J Gerontol A Biol Sci Med Sci 70(1) (2015) 33-43.
[173] J. Del Pozo-Cruz, E. Rodriguez-Bies, M. Ballesteros-Simarro, I. Navas-Enamorado, B.T. Tung, P. Navas, G. Lopez-Lluch, Physical activity affects plasma coenzyme Q10 levels differently in young and old humans, Biogerontology 15(2) (2014) 199-211.
[174] S. Onur, P. Niklowitz, A. Fischer, C.C. Metges, T. Grune, T. Menke, G. Rimbach, F. Doring, A comparative study into alterations of coenzyme Q redox status in ageing pigs, mice, and worms, Biofactors 40(3) (2014) 346-54.
[175] A. Fischer, S. Onur, P. Niklowitz, T. Menke, M. Laudes, G. Rimbach, F. Doring, Coenzyme Q10 Status as a Determinant of Muscular Strength in Two Independent Cohorts, PLoS One 11(12) (2016) e0167124.
[176] T. Nystrom, J. Yang, M. Molin, Peroxiredoxins, gerontogenes linking aging to genome instability and cancer, Genes Dev 26(18) (2012) 2001-8.
[177] J.G. de la Asuncion, A. Millan, R. Pla, L. Bruseghini, A. Esteras, F.V. Pallardo, J. Sastre, J. Vina, Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA, FASEB J 10(2) (1996) 333-8.
[178] R.S. Sohal, L. Arnold, W.C. Orr, Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/NADP+ and NADH/NAD+ in Drosophila melanogaster, Mech Ageing Dev 56(3) (1990) 223-35.
[179] D. Ghosh, K.R. Levault, G.J. Brewer, Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons, Aging Cell 13(4) (2014) 631-40.
[180] G. Landis, J. Shen, J. Tower, Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in Drosophila melanogaster, Aging (Albany NY) 4(11) (2012) 768-89.
[181] C. Lennicke, H.M. Cocheme, Redox signalling and ageing: insights from Drosophila, Biochem Soc Trans 48(2) (2020) 367-377.
[182] L. Bleier, I. Wittig, H. Heide, M. Steger, U. Brandt, S. Drose, Generator-specific targets of mitochondrial reactive oxygen species, Free Radic Biol Med 78 (2015) 1-10.
[183] K.E. Menger, A.M. James, H.M. Cocheme, M.E. Harbour, E.T. Chouchani, S. Ding, I.M. Fearnley, L. Partridge, M.P. Murphy, Fasting, but Not Aging, Dramatically Alters the Redox Status of Cysteine Residues on Proteins in Drosophila melanogaster, Cell reports 11(12) (2015) 1856-65.
[184] M. Sorensen, A. Sanz, J. Gomez, R. Pamplona, M. Portero-Otin, R. Gredilla, G. Barja, Effects of fasting on oxidative stress in rat liver mitochondria, Free Radic Res 40(4) (2006) 339-47.
[185] H. Xiao, M.P. Jedrychowski, D.K. Schweppe, E.L. Huttlin, Q. Yu, D.E. Heppner, J. Li, J. Long, E.L. Mills, J. Szpyt, Z. He, G. Du, R. Garrity, A. Reddy, L.P. Vaites, J.A. Paulo, T. Zhang, N.S. Gray, S.P. Gygi, E.T. Chouchani, A Quantitative Tissue-Specific Landscape of Protein Redox Regulation during Aging, Cell 180(5) (2020) 968-983 e24.
[186] R. Stefanatos, A. Sanz, The role of mitochondrial ROS in the aging brain, FEBS Lett 592(5) (2018) 743-758.
[187] A. Hiona, A. Sanz, G.C. Kujoth, R. Pamplona, A.Y. Seo, T. Hofer, S. Someya, T. Miyakawa, C. Nakayama, A.K. Samhan-Arias, S. Servais, J.L. Barger, M. Portero-Otin, M. Tanokura, T.A. Prolla, C. Leeuwenburgh, Mitochondrial DNA mutations induce mitochondrial

dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice, PLoS One 5(7) (2010) e11468.
[188] Y. Wang, D. Oxer, S. Hekimi, Mitochondrial function and lifespan of mice with controlled ubiquinone biosynthesis, Nature communications 6 (2015) 6393.
[189] S.B. Lee, I.H. Bae, Y.S. Bae, H.D. Um, Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death, J Biol Chem 281(47) (2006) 36228-35.
[190] J. Vasquez-Vivar, B. Kalyanaraman, P. Martasek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo, K.A. Pritchard, Jr., Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors, Proc Natl Acad Sci U S A 95(16) (1998) 9220-5.
[191] J.S. McNally, M.E. Davis, D.P. Giddens, A. Saha, J. Hwang, S. Dikalov, H. Jo, D.G. Harrison, Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress, Am J Physiol Heart Circ Physiol 285(6) (2003) H2290-7.
[192] J.S. McNally, A. Saxena, H. Cai, S. Dikalov, D.G. Harrison, Regulation of xanthine oxidoreductase protein expression by hydrogen peroxide and calcium, Arterioscler Thromb Vasc Biol 25(8) (2005) 1623-8.
[193] R. Radi, A. Cassina, R. Hodara, C. Quijano, L. Castro, Peroxynitrite reactions and formation in mitochondria, Free Radic Biol Med 33(11) (2002) 1451-64.
[194] C. Vida, S. Rodriguez-Teres, V. Heras, I. Corpas, M. De la Fuente, E. Gonzalez, The aged-related increase in xanthine oxidase expression and activity in several tissues from mice is not shown in long-lived animals, Biogerontology 12(6) (2011) 551-64.
[195] C.D. Pandya, B. Lee, H.A. Toque, B. Mendhe, R.T. Bragg, B. Pandya, R.T. Atawia, C. Isales, M. Hamrick, R.W. Caldwell, S. Fulzele, Age-Dependent Oxidative Stress Elevates Arginase 1 and Uncoupled Nitric Oxide Synthesis in Skeletal Muscle of Aged Mice, Oxid Med Cell Longev 2019 (2019) 1704650.
[196] D.R. Green, L. Galluzzi, G. Kroemer, Mitochondria and the autophagy-inflammation-cell death axis in organismal aging, Science 333(6046) (2011) 1109-12.
[197] T. Grune, T. Jung, K. Merker, K.J. Davies, Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease, Int J Biochem Cell Biol 36(12) (2004) 2519-30.
[198] A. Simonsen, R.C. Cumming, A. Brech, P. Isakson, D.R. Schubert, K.D. Finley, Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila, Autophagy 4(2) (2008) 176-84.
[199] L.R. Lapierre, C.D. De Magalhaes Filho, P.R. McQuary, C.C. Chu, O. Visvikis, J.T. Chang, S. Gelino, B. Ong, A.E. Davis, J.E. Irazoqui, A. Dillin, M. Hansen, The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans, Nature communications 4 (2013) 2267.
[200] J.O. Pyo, S.M. Yoo, H.H. Ahn, J. Nah, S.H. Hong, T.I. Kam, S. Jung, Y.K. Jung, Overexpression of Atg5 in mice activates autophagy and extends lifespan, Nature communications 4 (2013) 2300.
[201] D. Ryu, L. Mouchiroud, P.A. Andreux, E. Katsyuba, N. Moullan, A.A. Nicolet-Dit-Felix, E.G. Williams, P. Jha, G. Lo Sasso, D. Huzard, P. Aebischer, C. Sandi, C. Rinsch, J. Auwerx, Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents, Nature medicine 22(8) (2016) 879-88.
[202] A. Rana, M.P. Oliveira, A.V. Khamoui, R. Aparicio, M. Rera, H.B. Rossiter, D.W. Walker, Promoting Drp1-mediated mitochondrial fission in midlife prolongs healthy lifespan of Drosophila melanogaster, Nature communications 8(1) (2017) 448.

[203] A. Rana, M. Rera, D.W. Walker, Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan, Proc Natl Acad Sci U S A 110(21) (2013) 8638-43.
[204] G.M. Silva, L.E. Netto, V. Simoes, L.F. Santos, F.C. Gozzo, M.A. Demasi, C.L. Oliveira, R.N. Bicev, C.F. Klitzke, M.C. Sogayar, M. Demasi, Redox control of 20S proteasome gating, Antioxid Redox Signal 16(11) (2012) 1183-94.
[205] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, Z. Elazar, Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4, EMBO J 38(10) (2019).
[206] B. Carroll, E.G. Otten, D. Manni, R. Stefanatos, F.M. Menzies, G.R. Smith, D. Jurk, N. Kenneth, S. Wilkinson, J.F. Passos, J. Attems, E.A. Veal, E. Teyssou, D. Seilhean, S. Millecamps, E.L. Eskelinen, A.K. Bronowska, D.C. Rubinsztein, A. Sanz, V.I. Korolchuk, Oxidation of SQSTM1/p62 mediates the link between redox state and protein homeostasis, Nature communications 9(1) (2018) 256.
[207] S.L. Rea, V. Majcher, M.S. Searle, R. Layfield, SQSTM1 mutations--bridging Paget disease of bone and ALS/FTLD, Exp Cell Res 325(1) (2014) 27-37.
[208] S.M. Poulose, D.F. Bielinski, B. Shukitt-Hale, Walnut diet reduces accumulation of polyubiquitinated proteins and inflammation in the brain of aged rats, J Nutr Biochem 24(5) (2013) 912-9.
[209] S. Ma, I.Y. Attarwala, X.Q. Xie, SQSTM1/p62: A Potential Target for Neurodegenerative Disease, ACS Chem Neurosci 10(5) (2019) 2094-2114.
[210] L. Fernandez-Mosquera, K.F. Yambire, R. Couto, L. Pereyra, K. Pabis, A.H. Ponsford, C.V. Diogo, M. Stagi, I. Milosevic, N. Raimundo, Mitochondrial respiratory chain deficiency inhibits lysosomal hydrolysis, Autophagy 15(9) (2019) 1572-1591.
[211] U.T. Brunk, A. Terman, The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis, European journal of biochemistry / FEBS 269(8) (2002) 1996-2002.
[212] V.N. Gladyshev, Aging: progressive decline in fitness due to the rising deleteriome adjusted by genetic, environmental, and stochastic processes, Aging Cell 15(4) (2016) 594-602.
[213] M. Ogrodnik, H. Salmonowicz, V.N. Gladyshev, Integrating cellular senescence with the concept of damage accumulation in aging: Relevance for clearance of senescent cells, Aging Cell 18(1) (2019) e12841.
[214] X. Zheng, L. Boyer, M. Jin, J. Mertens, Y. Kim, L. Ma, L. Ma, M. Hamm, F.H. Gage, T. Hunter, Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation, eLife 5 (2016).
[215] C.D. Folmes, T.J. Nelson, A. Martinez-Fernandez, D.K. Arrell, J.Z. Lindor, P.P. Dzeja, Y. Ikeda, C. Perez-Terzic, A. Terzic, Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming, Cell Metab 14(2) (2011) 264-71.
[216] D.A. Sinclair, P. Oberdoerffer, The ageing epigenome: damaged beyond repair?, Ageing Res Rev 8(3) (2009) 189-98.
[217] L.N. Booth, A. Brunet, The Aging Epigenome, Mol Cell 62(5) (2016) 728-44.
[218] S. Horvath, DNA methylation age of human tissues and cell types, Genome Biol 14(10) (2013) R115.
[219] E. Maranzana, G. Barbero, A.I. Falasca, G. Lenaz, M.L. Genova, Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I, Antioxid Redox Signal 19(13) (2013) 1469-80.

[220] J.G. Wood, S. Hillenmeyer, C. Lawrence, C. Chang, S. Hosier, W. Lightfoot, E. Mukherjee, N. Jiang, C. Schorl, A.S. Brodsky, N. Neretti, S.L. Helfand, Chromatin remodeling in the aging genome of Drosophila, Aging Cell 9(6) (2010) 971-8.
[221] X. Liu, J. Secombe, The Histone Demethylase KDM5 Activates Gene Expression by Recognizing Chromatin Context through Its PHD Reader Motif, Cell reports 13(10) (2015) 2219-31.
[222] S.D. Pletcher, S.J. Macdonald, R. Marguerie, U. Certa, S.C. Stearns, D.B. Goldstein, L. Partridge, Genome-wide transcript profiles in aging and calorically restricted Drosophila melanogaster, Curr Biol 12(9) (2002) 712-23.
[223] A.P. Gomes, N.L. Price, A.J. Ling, J.J. Moslehi, M.K. Montgomery, L. Rajman, J.P. White, J.S. Teodoro, C.D. Wrann, B.P. Hubbard, E.M. Mercken, C.M. Palmeira, R. de Cabo, A.P. Rolo, N. Turner, E.L. Bell, D.A. Sinclair, Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging, Cell 155(7) (2013) 1624-38.
[224] E.F. Fang, M. Scheibye-Knudsen, L.E. Brace, H. Kassahun, T. SenGupta, H. Nilsen, J.R. Mitchell, D.L. Croteau, V.A. Bohr, Defective mitophagy in XPA via PARP-1 hyperactivation and NAD(+)/SIRT1 reduction, Cell 157(4) (2014) 882-896.
[225] E.F. Fang, Y. Hou, S. Lautrup, M.B. Jensen, B. Yang, T. SenGupta, D. Caponio, R. Khezri, T.G. Demarest, Y. Aman, D. Figueroa, M. Morevati, H.J. Lee, H. Kato, H. Kassahun, J.H. Lee, D. Filippelli, M.N. Okur, A. Mangerich, D.L. Croteau, Y. Maezawa, C.A. Lyssiotis, J. Tao, K. Yokote, T.E. Rusten, M.P. Mattson, H. Jasper, H. Nilsen, V.A. Bohr, NAD(+) augmentation restores mitophagy and limits accelerated aging in Werner syndrome, Nature communications 10(1) (2019) 5284.
[226] L. Fontana, L. Partridge, Promoting health and longevity through diet: from model organisms to humans, Cell 161(1) (2015) 106-118.
[227] M.G. Vizioli, T. Liu, K.N. Miller, N.A. Robertson, K. Gilroy, A.B. Lagnado, A. Perez-Garcia, C. Kiourtis, N. Dasgupta, X. Lei, P.J. Kruger, C. Nixon, W. Clark, D. Jurk, T.G. Bird, J.F. Passos, S.L. Berger, Z. Dou, P.D. Adams, Mitochondria-to-nucleus retrograde signaling drives formation of cytoplasmic chromatin and inflammation in senescence, Genes Dev 34(5-6) (2020) 428-445.
[228] J.G. Wood, B.C. Jones, N. Jiang, C. Chang, S. Hosier, P. Wickremesinghe, M. Garcia, D.A. Hartnett, L. Burhenn, N. Neretti, S.L. Helfand, Chromatin-modifying genetic interventions suppress age-associated transposable element activation and extend life span in Drosophila, Proc Natl Acad Sci U S A 113(40) (2016) 11277-11282.
[229] T.J. Maures, E.L. Greer, A.G. Hauswirth, A. Brunet, The H3K27 demethylase UTX-1 regulates C. elegans lifespan in a germline-independent, insulin-dependent manner, Aging Cell 10(6) (2011) 980-90.
[230] L. Garcia-Prat, M. Martinez-Vicente, E. Perdiguero, L. Ortet, J. Rodriguez-Ubreva, E. Rebollo, V. Ruiz-Bonilla, S. Gutarra, E. Ballestar, A.L. Serrano, M. Sandri, P. Munoz-Canoves, Autophagy maintains stemness by preventing senescence, Nature 529(7584) (2016) 37-42.
[231] T. Eisenberg, M. Abdellatif, S. Schroeder, U. Primessnig, S. Stekovic, T. Pendl, A. Harger, J. Schipke, A. Zimmermann, A. Schmidt, M. Tong, C. Ruckenstuhl, C. Dammbrueck, A.S. Gross, V. Herbst, C. Magnes, G. Trausinger, S. Narath, A. Meinitzer, Z. Hu, A. Kirsch, K. Eller, D. Carmona-Gutierrez, S. Buttner, F. Pietrocola, O. Knittelfelder, E. Schrepfer, P. Rockenfeller, C. Simonini, A. Rahn, M. Horsch, K. Moreth, J. Beckers, H. Fuchs, V. Gailus-Durner, F. Neff, D. Janik, B. Rathkolb, J. Rozman, M.H. de Angelis, T. Moustafa, G. Haemmerle, M. Mayr, P. Willeit, M. von Frieling-Salewsky, B. Pieske, L. Scorrano, T. Pieber,

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[232] S.E. Schriner, N.J. Linford, G.M. Martin, P. Treuting, C.E. Ogburn, M. Emond, P.E. Coskun, W. Ladiges, N. Wolf, H. Van Remmen, D.C. Wallace, P.S. Rabinovitch, Extension of murine life span by overexpression of catalase targeted to mitochondria, Science 308(5730) (2005) 1909-11.
[233] S.N. Radyuk, K. Michalak, V.I. Klichko, J. Benes, I. Rebrin, R.S. Sohal, W.C. Orr, Peroxiredoxin 5 confers protection against oxidative stress and apoptosis and also promotes longevity in Drosophila, Biochem J 419(2) (2009) 437-45.
[234] W. De Haes, L. Frooninckx, R. Van Assche, A. Smolders, G. Depuydt, J. Billen, B.P. Braeckman, L. Schoofs, L. Temmerman, Metformin promotes lifespan through mitohormesis via the peroxiredoxin PRDX-2, Proc Natl Acad Sci U S A 111(24) (2014) E2501-9.
[235] H.R. Massie, V.R. Aiello, T.R. Williams, Iron accumulation during development and ageing of Drosophila, Mech Ageing Dev 29(2) (1985) 215-20.
[236] H.R. Massie, V.R. Aiello, V. Banziger, Iron accumulation and lipid peroxidation in aging C57BL/6J mice, Exp Gerontol 18(4) (1983) 277-85.
[237] A. Picca, R.T. Mankowski, G. Kamenov, S.D. Anton, T.M. Manini, T.W. Buford, S.K. Saini, R. Calvani, F. Landi, R. Bernabei, E. Marzetti, C. Leeuwenburgh, Advanced Age Is Associated with Iron Dyshomeostasis and Mitochondrial DNA Damage in Human Skeletal Muscle, Cells 8(12) (2019).
[238] A.Y. Seo, J. Xu, S. Servais, T. Hofer, E. Marzetti, S.E. Wohlgemuth, M.D. Knutson, H.Y. Chung, C. Leeuwenburgh, Mitochondrial iron accumulation with age and functional consequences, Aging Cell 7(5) (2008) 706-16.
[239] H.R. Massie, V.R. Aiello, T.R. Williams, Inhibition of iron absorption prolongs the life span of Drosophila, Mech Ageing Dev 67(3) (1993) 227-37.
[240] A.P. Runko, A.J. Griswold, K.T. Min, Overexpression of frataxin in the mitochondria increases resistance to oxidative stress and extends lifespan in Drosophila, FEBS Lett 582(5) (2008) 715-9.

Figure Legends.

Figure 1. A four stages plan to determine where ROS are produced and test their impact on lifespan (or other phenotypes). A. Identify a physiological phenomenon to be studied, e.g. differences in lifespan between control and experimental group. B. Determine whether ROS are involved measuring differences between groups. The cartoon depicts ROS measurements in the fly brain C. Identify the generator of ROS using specific inhibitors and genetic approaches to specifically neutralize the generation of ROS from the proposed ROS generator. The cartoon shows how CI ROS production can be altered by rotenone, S1QELs or using RNAi against specific subunits of a respiratory complex. D. Suppress ROS production using inhibitors of the ROS generator (if possible) or targeted antioxidants, test whether the former affects the studied phenotype, e.g. test whether lifespan differences among the experimental and control groups disappear.

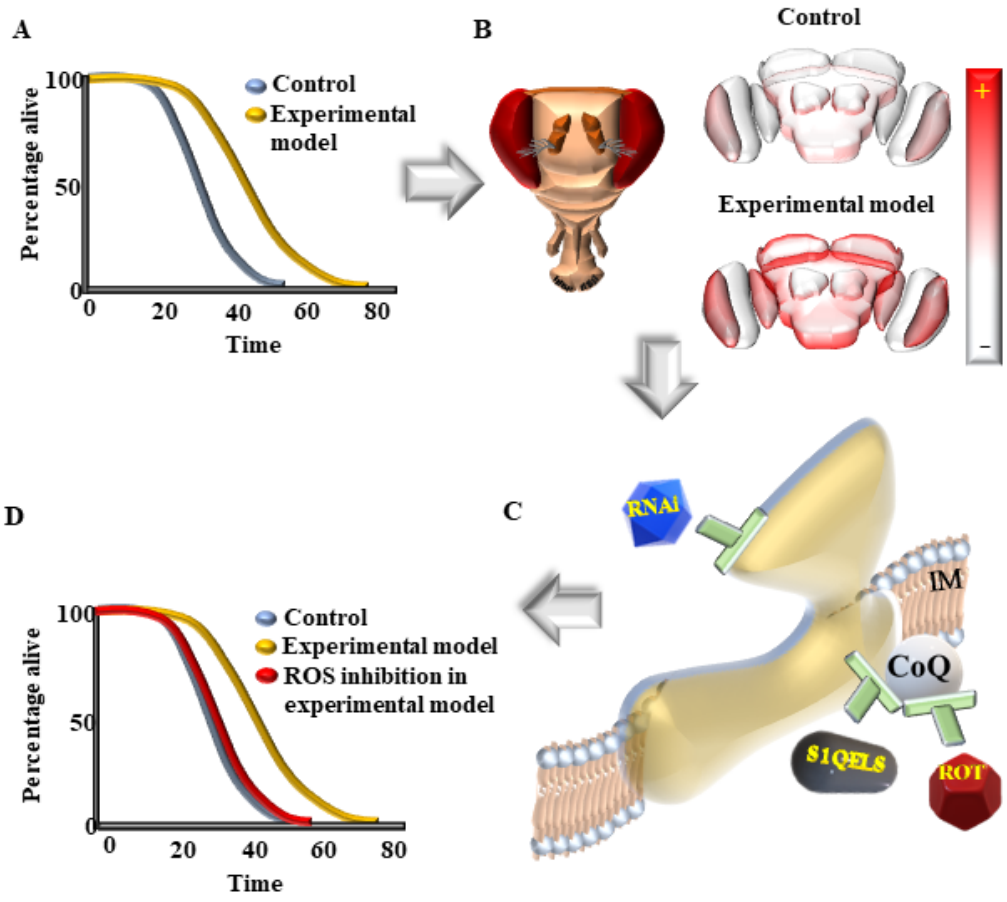
Figure 2. Effects of alternative respiratory enzymes, NDI1 and AOX, on the redox state of CoQ. NDI1 gives electrons to CoQ reducing it to $CoQH_2$, increasing the $CoQH_2$:CoQ ratio. This process favours the generation of ROS-RET at CI. Thus, NDI1 restores production of ROS by CIII when CI is blocked with rotenone or metformin. NDI1 rescues alterations in CI (block or depletion), alleviates Parkinson's disease symptoms in model organisms, protects against Ischemia/Reperfusion injury in rodents and extends the lifespan of flies. Conversely, AOX takes electrons from a highly reduced $CoQH_2$ and reduces oxygen to water. In doing so, it prevents both ROS-RET as well as the accumulation of $CoQ^{\cdot}$ at CIII. AOX can by-pass CIII and CIV defects, rescue developmental defects in flies and protects against smoke damage and LPS lethality in rodent models of disease.

Figure 3. Physiological processes regulated by CI ROS. Various processes have been shown to be regulated by CI ROS produced via RET: 1) Myoblast differentiation, 2) Cell death caused by ischemia-reperfusion (IR) during a heart attack, 3) Oxygen sensing by the glomus cells of the carotid body, 4) Levels of CI and assembly of the respirasome, 5) Macrophage reprogramming in response to bacterial infection, 6) Lifespan of *Drosophila melanogaster*, 7) Stress adaptation in response to thermal, hypoxic and hyperoxic stress. It is unclear where ROS are produced within CI during RET and both the quinone-binding site (IQ) and flavin (IF) have been reported as ROS generators. To demonstrate that ROS are produced via RET at CI one or more of the following controls needs to be performed: i) use of an IQ inhibitor such as rotenone, ii) use of CII inhibitors such as malonate, iii) knockdown/out of CI/CII subunits, iv) dissipating the proton motive force (pmf) by e.g. FCCP, v) expression of an alternative oxidase (AOX) to maintain oxidation of the CoQ pool vi) use of S1QELs.

Figure 4. Physiological processes regulated by CIII ROS. Different processes have been shown to be regulated by CIII ROS produced at the IIIQo site: 1) Hypoxia adaptation in cells, 2) Acute hypoxia pulmonary vasoconstriction (AHPV), 3) T-cell activation in response to the presence of antigens, 4) Tissue repair mediated by TGF-B signalling (in excess can cause fibrosis), 5) Adipocytes differentiation, 6) Tumorigenesis, 7) Damage induced by pollution or smoking. ROS are produced as a consequence of the formation of $CoQ^{\cdot}$ during the Q-cycle. To demonstrate that ROS are produced by CIII one or more of the following controls need to be performed: i) use of an IIIQo inhibitor such as myxothiazol, ii) knockdown/out of RISP protein, iii) expression of AOX, iv) use of CI inhibitors in combination with NDI1 expression and v) application of S3QELs

Figure 5. Effects of ageing on mitochondrial redox signalling. Young mitochondria have enough supply of NAD+, and oxygen consumption is high, allowing the cells to produce ATP efficiently. CI and CIII are assembled into supercomplexes that generate ROS in a controlled manner to produce redox signals. A moderate amount of superoxide is produced, and this is immediately dismutated to hydrogen peroxide. Hydrogen peroxide is the ROS messenger that transmits information by selective oxidation of specific cysteine residues within target proteins. For example, peroxiredoxins are selectively oxidized by hydrogen peroxide and then they can oxidize other proteins to continue the signalling process. Similarly, CIII ROS can oxidize, assisted by peroxiredoxins or independently, critical proteins involved in cellular homeostasis such as HIF1α, NFkB or SQSTM1/p62. The situation becomes much less clear during ageing. Old mitochondria do not have sufficient NAD+, consume less oxygen and produce less ATP. Supercomplexes are disassembled and ROS production is high and uncontrolled. High levels of superoxide and hydrogen peroxide react with free metals, such as iron, to generate hiROS like hydroxyl radicals. Redox signalling is interrupted and dormant ROS generators such as nitrogen oxide synthase and xanthine oxidase become active, generating a significant amount of ROS further distorting redox signalling.

Graphical abstract


A
Percentage alive
100
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0
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60
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Time
Control
Experimental model
B
Control
Experimental model
+
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C
RNAi
IM
CoQ
S1QELS
ROT
D
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100
50
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60
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Time
Control
Experimental model
ROS inhibition in experimental model

Figure 1

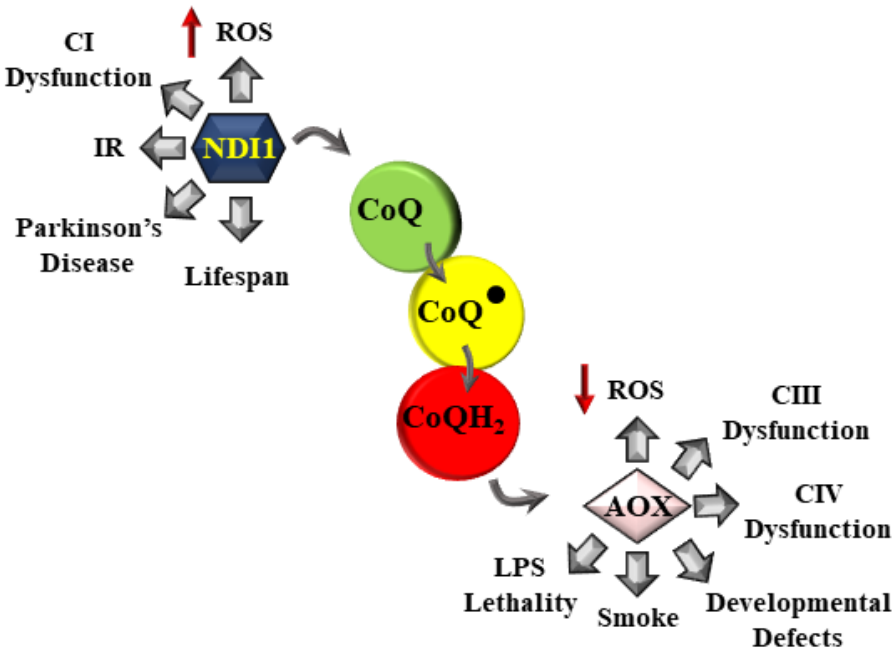

CI
Dysfunction
ROS
IR
NDI1
Parkinson's
Disease
Lifespan
CoQ
CoQ
CoQH2
ROS
CIII
Dysfunction
AOX
CIV
Dysfunction
LPS
Lethality
Smoke
Developmental
Defects

Figure 2

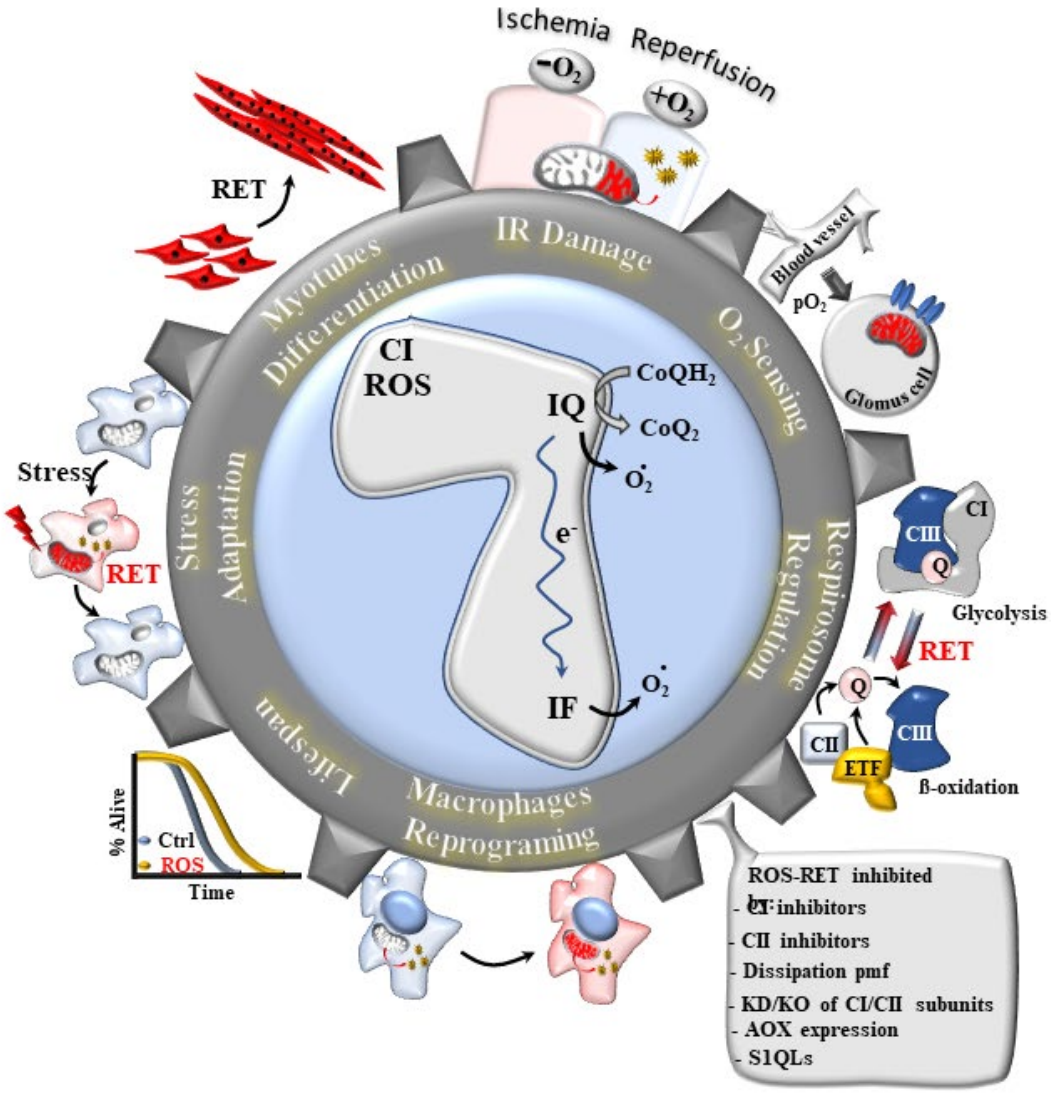
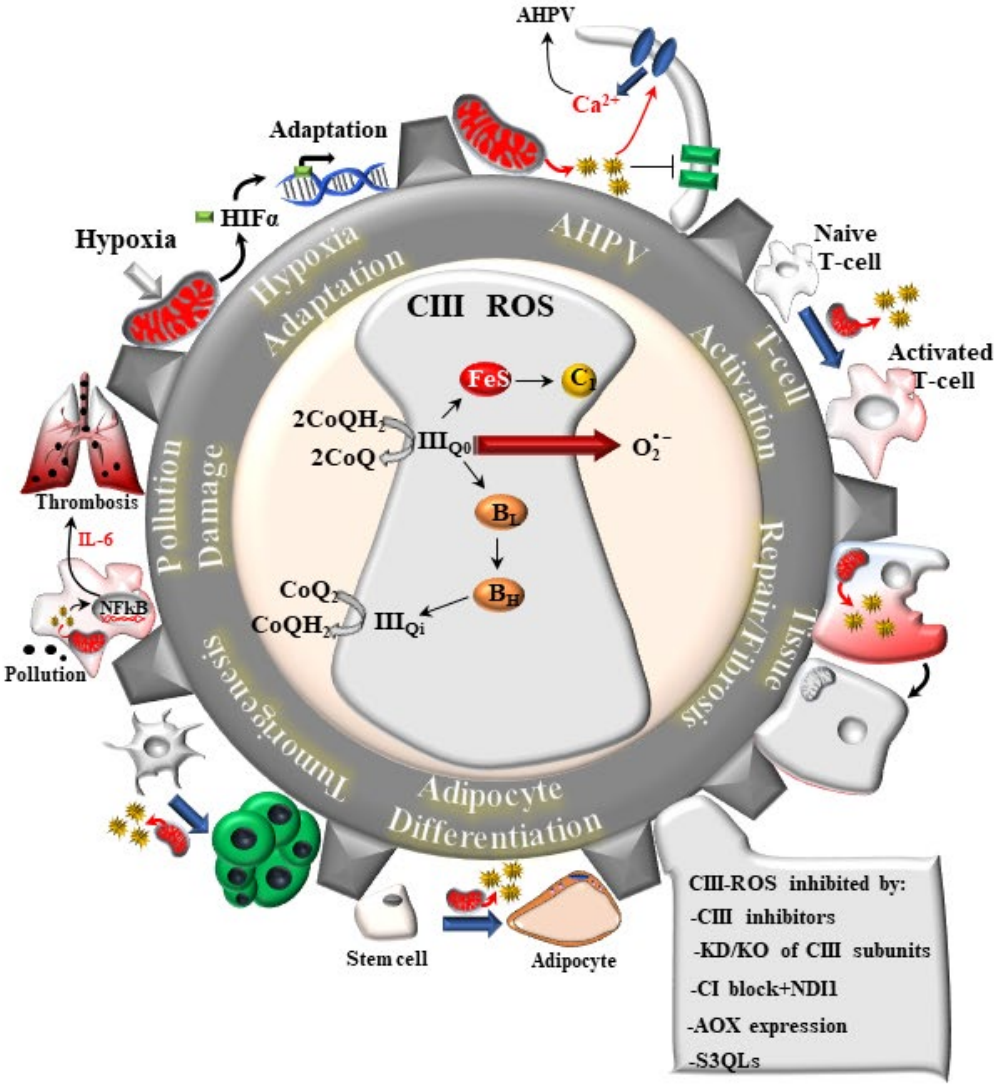

Ischemia Reperfusion
-O2
+O2
RET
IR Damage
Myotubes Differentiation
O2 Sensing
Respirosome Regulation
Macrophages Reprograming
Lifespan
Stress Adaptation
CI
ROS
IQ
CoQH2
CoQ2
O2·
e-
IF
O2·
Blood vessel
pO2
Glomus cell
Stress
RET
CI
CIII
Q
Glycolysis
RET
Q
CII
CIII
ETF
ß-oxidation
% Alive
Ctrl
ROS
Time
ROS-RET inhibited
- CI inhibitors
- CII inhibitors
- Dissipation pmf
- KD/KO of CI/CII subunits
- AOX expression
- S1QLs

Figure 3


AHPV
Ca2+
Adaptation
HIFα
Hypoxia
Hypoxia Adaptation
AHPV
Naive T-cell
Activated T-cell
T-cell Activation
CIII ROS
FeS
C1
2CoQH2
2CoQ
IIIQ0
O2•−
BL
BH
CoQ2
CoQH2
IIIQi
Repair/Fibrosis Tissue
Thrombosis
IL-6
NFkB
Pollution
Pollution Damage
Tumorigenesis
Adipocyte Differentiation
Stem cell
Adipocyte
CIII-ROS inhibited by:
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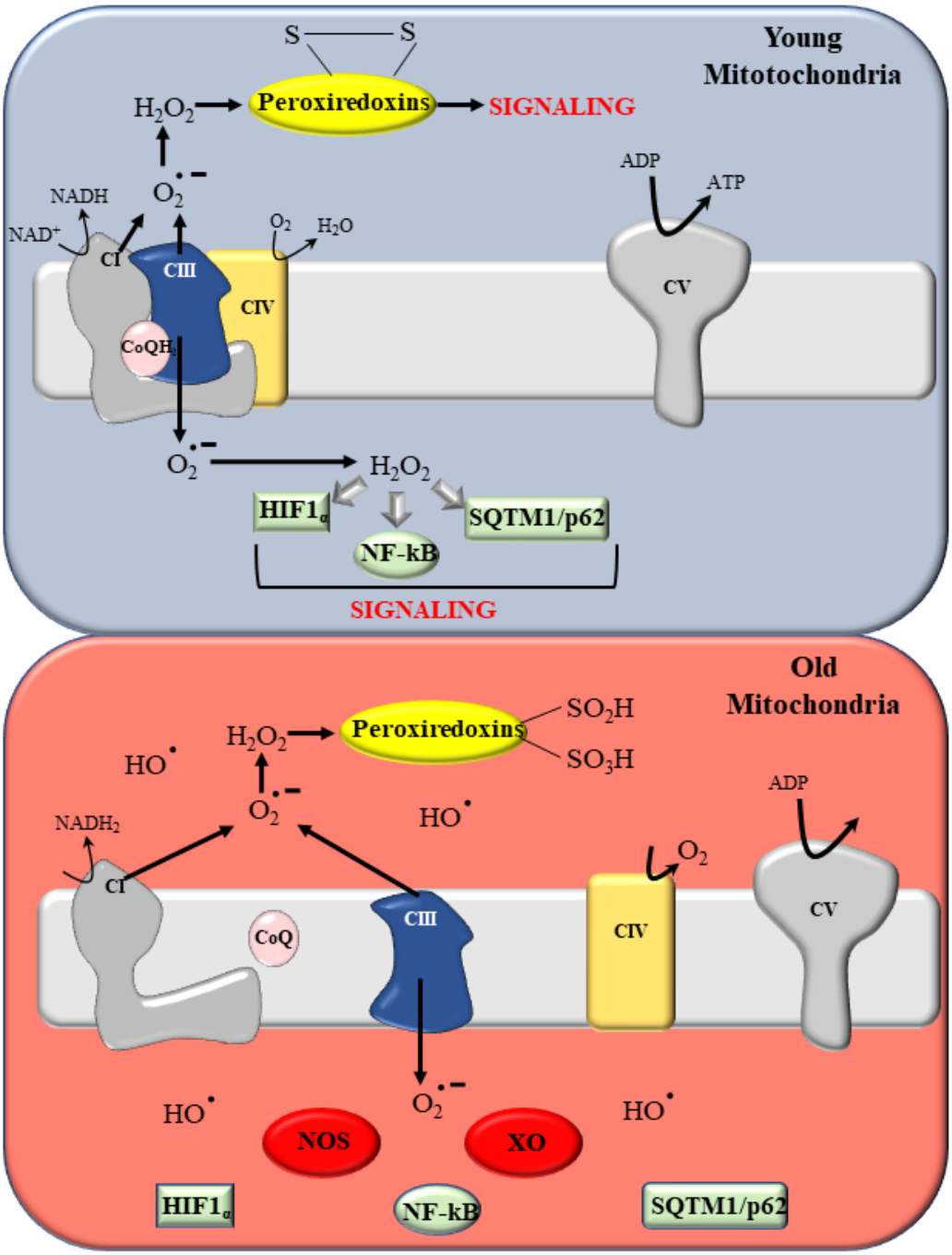
-KD/KO of CIII subunits
-CI block+NDI1
-AOX expression
-S3QLs

Figure 4


Young
Mitotochondria
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S
H_2O_2
Peroxiredoxins
SIGNALING
ADP
ATP
NADH
$O_2^{\bullet-}$
NAD^+
CI
CIII
O_2
H_2O
CIV
CV
$CoQH_2$
$O_2^{\bullet-}$
H_2O_2
$HIF1_\alpha$
SQTM1/p62
NF-kB
SIGNALING
Old
Mitochondria
H_2O_2
Peroxiredoxins
SO_2H
SO_3H
$HO^\bullet$
$O_2^{\bullet-}$
$HO^\bullet$
ADP
$NADH_2$
CI
CoQ
CIII
O_2
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CV
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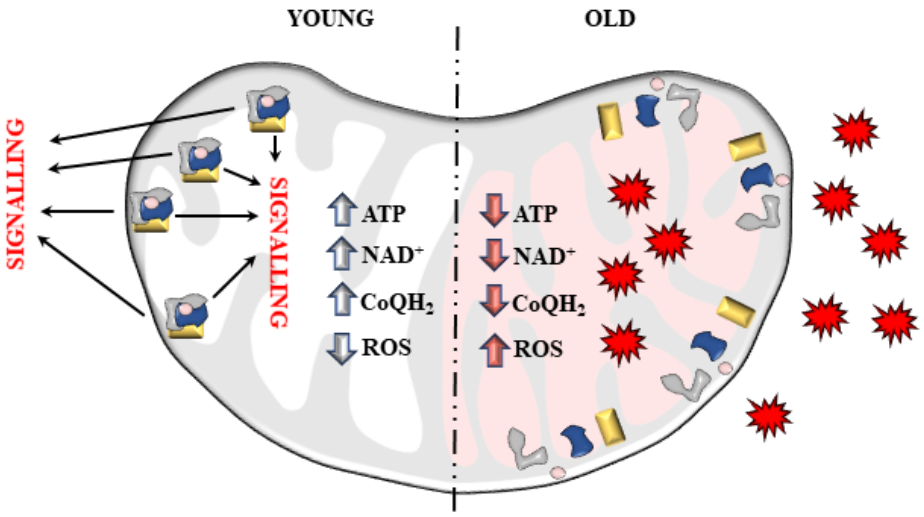
XO
$HIF1_\alpha$
NF-kB
SQTM1/p62

Figure 5



Graphical abstract